Universidade do Minho
Escola de Engenharia

Bartolomeu Warlene Silva de Souza

Characterisation of new hydrocolloids to be used as food coatings and integration of their application with ohmic heating

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Characterisation of new hydrocolloids to be used as food coatings and integration of their application with ohmic heating

Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho efectuado sob a orientação do
Professor Doutor José A. Couto Teixeira
e do
Professor Doutor António Augusto Vicente

Setembro de 2010

"Ando devagar porque já tive pressa
Levo esse sorriso porque já chorei demais
Hoje me sinto mais forte, mais feliz quem sabe
Só levo a certeza de que muito pouco eu sei, (...)
É preciso amor pra poder pulsar,
É preciso paz pra poder sorrir,
É preciso a chuva para florir
Penso que cumprir a vida seja simplesmente
Compreender a marcha e ir tocando em frente (...)
Todo mundo ama um dia
Todo mundo chora, um dia a gente chega e no outro vai embora
Cada um de nós compõe a sua história
Cada ser em si carrega o dom de ser capaz
De ser feliz(...)"

Almir Sater & Renato Teixeira

Aos meus grandes amores: Meu Pai, minha Mãe (*in memoriam*) e a Fábia,

Dedico

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Summary

The interest in the development of edible films and coatings for foods has grown considerably due to consumers demand for high quality foods and environmental concerns over the disposal of non-renewable food packaging materials. The potential of edible films to control water transfer, and to improve food quality and shelf life, has received increasing attention from researchers and industry. Currently, technologies utilizing electrical fields directly into food processing raised a significant interest in the food industry. Several of those are now being used on a commercial scale for processing of an extensive range of food products. The application of electric fields has also been addressed by researchers in the area of edible films and coatings.

In this context, the leitmotiv of this Thesis is to provide added-value to natural materials from renewable sources (algae from sea-farms and by-products from the fishing industry) with the more "at-large" objective of bringing development to underdeveloped regions of the World.

In particular, the research undertaken was directed to the chemical characterization and evaluation of the antioxidant activity of sulfated polysaccharides from the red seaweed *Gracilaria birdiae* (*Gb*), farmed in the north-eastern coast of Brazil. The work performed allowed to conclude that the sulfated polysaccharide from *Gb* is composed of galactose (65.4 %) and methyl derivatives 6-O-methyl-galactose (9.2 %) and in smaller quantities 3-O- and 4-O-methyl-galactose (0.33 %). This polysaccharide also presents a high content of 3,6-anhydrogalactose (25.6 %) and has a sulfate content of 8.4 %. The sulfated polysaccharide of *Gb* characterized by FTIR exhibits the characteristic bands of agarocolloids (at 1375 and 770 cm^{-1}). It has also been shown that the *Gb* sulfated polysaccharide is a promising agent to be evaluated for the application in the food industry, and that it presents a significant antioxidant activity. A study of the applications of this sulfated polysaccharide was performed and the possibility of using it as coating material on semi-hard cheese was found. The choice of the best coating was made taking into consideration its wettability, permeability and opacity properties.

A second objective was related with determining the effect of field strength on transport properties of chitosan coatings (obtained from lobster from the Cuban coasts). Four different field strengths were tested (50, 100, 150, 200 $V \cdot cm^{-1}$) and, for each electric field treatment, the water vapor, oxygen and carbon dioxide permeabilities of the films formed were determined, together with their color, opacity and solubility in water. Results showed that electric field had statistically significant effects on film's physical properties and structure. In general, the most pronounced effect of the field strength was

observed for treatments made at 100 Vcm^{-1} or higher, a positive correlation being found between the water vapor, oxygen and carbon dioxide permeability coefficients and field strength.

In the same perspective the effect of electric fields applied at different field strength values on mechanical and thermal properties of chitosan films/coatings was evaluated. XRD analyses indicated that electrically treated chitosan films exhibited a more ordered structure and a clearly higher crystallinity when compared with non-treated films, thus displaying significant effects on the value of the crystallinity index (CI). SEM micrographs evidenced that the surface morphology of chitosan films was influenced by the electric field. In fact, the electric field treatment led to a structure with more regular layers. The application of the electric field to chitosan film-forming solutions resulted in an increase of the tensile strength (ca. 9 %) and elongation-at-break (ca. 18 %) of the corresponding chitosan films. The reported results demonstrate that the application of an electric field to film-forming solutions of chitosan is an interesting instrument to taylor relevant properties of the films or coatings produced from them.

Finally, the effects of the application of chitosan coating with or without electric field treatment in sliced salmon stored in ice at 0 °C were analyzed. In a first stage, the surface properties of salmon fillets and the wetting capacity of the coatings on fish were evaluated. The best wettability results were obtained with 1 % chitosan solutions with a spreading coefficient (*Ws*) of -4.73 $mN \cdot m^{-1}$. For shelf-life analyses the fillets were coated and stored at 0 °C for 18 days. The control and the coated fish samples were analyzed periodically for total mesophilic count (TPC), pH, total volatile base nitrogen (TVB-N), trimethylamine (TMA), thiobarbituric acid (TBA) and ATP breakdown products (*K*-value). A significant reduction ($p < 0.05$) in pH and *K*-value starting from the sixth day until the end of analysis (day 18) and in TVB, TMA and TBA from the ninth until the last day, was observed for fish samples coated with chitosan, when compared to control samples. In terms of microbial growth, a slower increase in TPC was observed for the coated fish, indicating that chitosan-based coatings were effective in extending for 3 more days the shelf-life of salmon. These results demonstrate that chitosan-based coatings may be an alternative for extending the shelf-life of salmon fillets during storage at 0 °C. However, no significant differences ($p < 0.05$) were observed between chitosan coatings with or without electric field treatment.

In short, the sulfated polysaccharide of *Gb* is a promising coating-forming and antioxidant agent for applications in the food industry. The application of electric fields during chitosan coating solutions preparation may provide a novel method for production of coatings with taylored properties. However, further research is needed for a clearer understanding of the importance of these changes on real food systems. Important data have also been generated on the use of chitosan edible coatings on fish.

Resumo

Nos dias de hoje, é grande o interesse no desenvolvimento de filmes e revestimentos comestíveis ou biodegradáveis, principalmente devido à demanda por alimentos de alta qualidade, e preocupações ambientais sobre o descarte de materiais não renováveis de embalagem para alimentos. O potencial dos filmes comestíveis em controlar a transferência de vapor de água, melhorar a qualidade dos alimentos e do tempo de prateleira, tem recebido atenção dos pesquisadores e das indústrias. A utilização de tecnologias utilizando campos eléctricos directamente nos alimentos processados gerou um interesse significativo na indústria alimentar. Várias destas tecnologias são usadas em escala comercial no processamento de vários produtos alimentares. A aplicação de campos eléctricos também tem despertado o interesse de pesquisadores na área de filmes e revestimentos edíveis.

Neste contexto, o propósito da presente tese foi no sentido de oferecer um valor acrescentado aos materiais naturais de fontes renováveis (algas marinhas cultivadas e subprodutos da indústria da pesca) com um objectivo maior de levar o desenvolvimento para regiões subdesenvolvidos.

Em particular, a investigação realizada foi direccionada para a caracterização química e avaliação da actividade antioxidante de polissacarídeos sulfatados da alga vermelha *Gracilaria birdiae* (*Gb*). O trabalho realizado permitiu concluir que os polissacarídeos sulfatados de *Gb* são compostos de galactose (65,4 %) e derivados metil 6-O-metil-galactose (9,2 %) e em menores quantidades 3-O-e 4-O - metil-galactose (0,33 %), 3,6-anhydrogalactose (25,6 %) e um teor de sulfato de 8,4 %. As analises por FTIR apresentou bandas características de agarocolóides (a 1375 e 770 cm^{-1}). Também se demonstrou que o polissacarídeo sulfatado é um agente promissor para aplicação na indústria de alimentos, e que apresenta uma actividade antioxidante significativa. Realizou-se um estudo das possíveis aplicações deste polissacarídeo. Em particular, estudou-se a possibilidade da sua utilização como cobertura em queijo semi-rígido, tendo a escolha do melhor revestimento sido feita considerando a sua molhabilidade, permeabilidade a gases e opacidade.

Um segundo objectivo foi determinar o efeito da intensidade de campos eléctricos nas permeabilidades dos revestimentos de quitosano (obtidos a partir de lagosta capturada em águas costeiras de Cuba). Testaram-se quatro intensidades de campo diferentes (50, 100, 150, 200 V $.cm^{-1}$) e, para cada uma delas, determinaram-se as permeabilidade ao vapor de água, oxigénio e dióxido de carbono dos filmes formados, juntamente com a respectiva cor, opacidade e solubilidade em água. Os resultados mostraram que a aplicação do campo eléctrico alterou a estrutura e as propriedades físicas dos filmes. Em geral, o efeito mais pronunciado da intensidade do campo observou-se para os tratamentos

realizados a 100 Vcm^{-1} ou mais, encontrando-se uma correlação positiva entre as permeabilidades e a intensidade do campo. Em uma mesma perspectiva avaliou-se o efeito de campos eléctricos sobre as propriedades mecânicas e térmicas de filmes de quitosano. Os resultados de difracção de raios X indicaram que os filmes electricamente tratados exibiram uma estrutura mais ordenada e uma cristalinidade claramente superior quando comparados com os filmes não-tratados, mostrando efeitos significativos no valor do índice de cristalinidade (IC). A observação de amostras por microscopia electrónica de varrimento evidenciou que a morfologia da superfície dos filmes de quitosano foi influenciada pelo campo eléctrico, tendo originado estruturas com camadas mais regulares. Amostras electricamente tratadas apresentaram um aumento da resistência à tracção (cerca de 9 %) e do alongamento, (cerca de 18 %) dos filmes de quitosano.

No seguimento estudaram-se os efeitos da aplicação dos revestimentos de quitosano anteriormente desenvolvidos, com ou sem tratamento eléctrico, em filetes de salmão resfriado, armazenados a 0 °C. Numa primeira fase, avaliaram-se as propriedades de superfície dos filetes e a capacidade molhante dos revestimentos. Os melhores resultados de molhabilidade, foram obtidos com soluções a 1 % de quitosano com um coeficiente de espalhamento de -4,73 mNm^{-1}. Para análise do tempo de prateleira, os filetes foram revestidos e armazenados a 0 °C por 18 dias. As amostras de controlo e as amostras de peixe revestidas foram analisadas periodicamente para contagem de mesófilos totais (TPC), pH, e para a quantificação das bases nitrogenadas voláteis totais (N-BVT), trimetilamina (TMA), ácido tiobarbitúrico (TBA) e produtos de degradação do ATP (*K*-valor). Observou-se uma redução significativa para amostras de peixe revestido com quitosano, quando comparadas com amostras controle ($p < 0,05$) no pH e no *K*-valor partir do sexto dia até o final de análise (dia 18) e nos testes de N-BVT, TMA e TBA a partir do nono até o último dia. Em termos de crescimento microbiano, observou-se um aumento mais lento do TPC para os peixes revestidos, prorrogando por mais três dias o prazo de validade do salmão. Os revestimentos de quitosano podem constituir uma alternativa para prolongar o tempo de prateleira de filetes de salmão durante o armazenamento a 0 °C. Não foram observadas diferenças significativas ($p < 0,05$) entre o revestimento de quitosano com e sem tratamento com campo eléctrico. Em resumo, o polissacarídeo sulfatado de *Gb* rico em actividade antioxidante, é um agente promissor na formação de revestimentos comestíveis. A aplicação de campos eléctricos durante a preparação de soluções de revestimento de quitosano podem fornecer uma nova metodologia para a produção de revestimentos comestíveis. Entretanto, mais pesquisas são necessárias para uma compreensão mais clara da importância destas mudanças nos sistemas alimentares. Dados importantes também foram geradas sobre o uso de revestimentos de quitosano em peixes.

List of Publications

This thesis is based on the following original articles:

Cerqueira, A.M., Souza, B.W.S., Martins, J.T., Vicente, A.A. (2010). Improved hydrocolloid-based edible coatings/films systems for food applications. In: Ashutosh Tiwari and Frank Columbus (Eds.). Carbohydrate Polymers: Development, properties and applications. New York, USA: Nova SciencePublishers, 2010, V. cap 14 (Accepted). [Chapter 2]

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Souza, B.W.S., Cerqueira, M.A., Bourbon, A.I., Pinheiro, A.C., Martins, J.T., Teixeira, J.A., Coimbra, M.A., Vicente, A.A. (2010). Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed *Gracilaria birdiae.* Food hydrocolloids. (Submitted). [Chapter 3]

Cerqueira, M.A., Lima, A.M., Souza, B.W.S., Teixeira, J.A., Moreira, R.A., Vicente, A.A.(2009) Funtional Polysaccharides as Edible coaings for cheese. Journal of Agricultural and Food Chemistry, 57(4), 1456, 1462. [Chapter 4]

Souza, B. W. S., Cerqueira, M. A., Casariego, A., Lima, A. M. P., Teixeira, J. A., Vicente, A. A. (2009). Effect of moderate electric fields in the permeation properties of chitosan coatings. Food Hydrocolloids, 23, 2110–2115. [Chapter 5]

Souza, B. W. S., Cerqueira, M. A., Martins, J. T., Casariego, A., Teixeira, J. A., Vicente, A. A. (2010). Influence of electric fields on the structure of chitosan edible coatings. Food Hydrocolloids, 24, 330-335. [Chapter 6]

Souza, B. W. S., Cerqueira, M. A., Ruiz, H.A., Casariego, A., Teixeira, J. A., Vicente, A. A. (2010). Effect of chitosan based coating on shelf-life of salmon (*Salmo solar*). Journal of Agricultural and Food Chemistry. (Accepted). [Chapter 7]

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List of general nomenclature

Abreviations

ADP- Adenosine monophosphate

AFM- Aomic force macroscopy

ATP - Adenosine triphosphate

BHA - Butylated hydroxyanisole

BHT- Butylated hydroxytoluene

CFU - Colony-forming units

CI – Cristallinity index

CO_2P- Carbon dioxide permeability

DPPH - 1,1-diphenyl-2-picrylhydrazyl

DSC - Differential scanning calorimetry

E – Elongation at break

FDA- Food and Drug administration

FTIR – Fourier transform infrared spectroscopy

Gb - *Gracilaria birdiae*

GPC – Gel permeation chromatography

HPLC – High performance liquid chromatography

Hx - Hypoxanthine

HxR - Inosine

IMP - Inosine monophosphate

K-value – ATP breakdown products

MDA - Malonaldehyde

O_2P- Oxygen permeability

PG - Propyl gallate

PHA – Polyhydroxyalkonoates

R.H- Relative humidity

SEM – Scanning electronic microscopy

TBA- 2- thiobarbituric acid

TBHQ - *tert*-butylhydroquinone

TCA - trichloroacetic acid

TMA - Trimethylamine

TPC – Total aerobic plate count

TS – Tensile strength

TVB - Total volatile base nitrogen

Wa – Adhesion coefficient

Wc – Cohesion coefficient

Ws – Spreading coefficient

WVP – Water vapor permeability

WVTR – Water vapor transmission rate

XRD – X-ray diffractions

Symbols

G'- storage modulus [pa]

G"- loss molulus [pa]

IC_{50}- concentration of substance needed to inhibit 50%

T- Temperature [°C]

T_m – Melting temperature

γ_{LV}- Tension solid-vapor

γ_{SL}-Tension solid-vapor

γ_{SV} - Tension solid-vapor

ΔH_m – Melting enthalphy

η - Viscosity [Pa.s]

θ - Contact angle

ω - Oscillatory frequency [rad/s]

Chapter 1 - Outline of the thesis

Edible films and coatings have received considerable attention in recent years because of their advantages over synthetic films. The main advantage of edible films over traditional synthetics is that they can be consumed with the packaged products. There is no package to dispose and even if the films are not consumed they could still contribute to the reduction of environmental pollution. Edible films are produced exclusively from renewable, edible ingredients and therefore are anticipated to degrade more readily than polymeric materials (Bourtoom 2008). Consequently improvements in the amount of knowledge on edible films and coatings, acquired through research and product development work, as well as advances in material science and processing technology have occurred. The potential of edible films to control water transfer and to improve food quality and shelf life, has received increasing attention from researchers and industry (Krochta 2002). However, the search for new renewable materials from natural sources is an important task when new functionalities are sought and/or new uses for less noble materials must be enforced for economical and environmental reasons.

In recent years, marine resources have attracted attention in the search for bioactive compounds to develop new drugs and healthy foods (Qi et al., 2005). In particular, seaweeds are a very important and commercially valuable resource for food, fodder, soil conditioners and pharmaceuticals (Yang et al., 2006). Brazilian coasts and seas, with their vast extension, are very rich in unexploited or under-exploited biological resources. Some local communities are now being supported by governmental and non-governmental projects, which aim at providing them with means of improving their life standards while practicing a sustainable exploitation of such resources. These include the cultivation and collection of algae in the northeast coast of the country. Such algae are already commercialized, but the aim is to improve their added-value by giving them a different use. The red marine alga *Gracilaria birdiae* has a great economic impact in Brazil due to agar production (Plastino, Ursi & Fuji, 2004). No studies have yet been performed on the chemical characterization of its polysaccharides or on potential higher added-value uses by the food industry.

Cuban coasts are very rich in crustaceous, namely lobsters, and therefore the fishing industry is a very important sector of activity. Furthermore, most of the lobster is locally processed and exported, their carapaces being a left over that can be used to obtain chitosan. In fact, a local project has been running for several years aiming at producing chitosan with a high degree of deacetylation. Given its economical relevance for the country, the project is now at the industrial level and the chitosan produced is being evaluated aiming at higher added-value applications e.g. in the food industry (Casariego et al., 2008).

The leitmotiv of this Thesis is therefore to provide added-value to natural materials from renewable sources with the more “at-large” objective of bringing development to underdeveloped regions of the

World.

Nowadays, technologies utilizing electrical fields directly into food processing raised a significant interest in the food industry. Several of those are now being used on a commercial scale for processing of an increasing range of food products. The application of electric fields has also been addressed by researchers in the area of edible films and coatings (Garcia et al., 2009; Lei et al., 2007).

In these contexts, the aim of this thesis was at first, to characterize chemically materials of natural origins, which can be used as edible coatings for foodstuffs and which can replace with advantages those actually in use. The research undertaken was directed to the chemical characterization and evaluation of the antioxidant activity of sulfated polysaccharides from the red seaweed *Gracilaria birdiae* (*Gb*). A study of the applications of this sulfated polysaccharide was performed and the possibility of using it as coating material on semi-hard cheese was explored. A second objective was to evaluate the effect of field strength in chitosan coating solutions and to characterize the coatings' transport properties, microstructure and mechanical properties, and to apply these coatings on food products (e.g. sliced salmon stored at 0 °C).

Based on these main objectives, this thesis was organized in eight chapters. Chapter 2 provides an overview of the state-of-the-art, Chapters 3 to 7 contain the main experimental results while Chapter 8 presents the general conclusions and futures perspectives.

In Chapter 2, the thesis starts with an overview of edible coating and films, the general materials used, film-forming mechanism followed by influence of electric fields in edible coatings/films properties.

The chemical characterization of sulfated polysaccharide from the red seaweed *Gracilaria birdiae* and their antioxidant properties is introduced in Chapter 3. The hot extraction of polysaccharide was studied. The monosaccharide composition obtained by reductive hydrolysis was analyzed by gas chromatography. The molar mass, FTIR analysis and rheological measurements were determined. Scavenging effects in vitro on DPPH and hydroxyl radials were analyzed.

In Chapter 4 the ability of sulfated polysaccharide from seaweed *Gracilaria birdiae* to be used as coatings for cheese is described. The surface properties of the cheese and the wetting capacity of the coatings on the cheese were determined. The three best solutions for polysaccharide were chosen,

further films were cast and permeabilities to water vapor, oxygen and carbon dioxide were determined, as well as opacity.

In Chapter 5 the effect of field strength on functional properties of chitosan coatings was evaluated. Four different field strengths were tested (50, 100, 150, 200 $V \cdot cm^{-1}$) and, for each electric field treatment, the water vapor, oxygen and carbon dioxide permeabilities of the films formed were determined, together with their color, opacity and solubility in water. The surface microstructure of the films was analyzed using atomic force microscopy (AFM).

The influence of electric fields on the structure of chitosan edible coatings is described in Chapter 6. Mechanical and thermal properties of chitosan films/coatings were analyzed when compared with non-treated films using X- ray diffraction (XRD) and Scanning electron microscopy (SEM).

Chapter 7 describes the effects of chitosan coating on shelf-life extension of *Salmo solar* fillets. The surface properties of the salmon fillets and the wetting capacity of the coatings on the fish surface were determined. The best coating-forming solution in terms of wettability was electrically treated, the fillets were coated with it and stored in ice (0 °C) for 18 days. Control and coated fish samples were analysed periodically for total mesophilic count (TPC), pH, total volatile base nitrogen (TVB-N), trimethylamine (TMA), thiobarbituric acid (TBA) and ATP breakdown products (*K*-value).

Overall conclusions and futures perspectives are presented in Chapter 8.

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Chapter 2 - General introduction

2.1 Polymer-based edible coatings/films

In the present moment, biopolymers have been slow to reach commercial maturity, due to the higher costs and less optimal physical properties when compared with conventional, synthetic plastics. In addition, there have not been sufficient incentives for downstream processors to incorporate those biodegradable materials into their products. About 150 million tons of plastics are produced annually all over the world, and their production and consumption continue to increase (Parra et al., 2004). The ecological impact of raw material resources used in manufacturing products and their ultimate disposal are relevant considerations in their design. Products designated, as "ecoefficient" are the new generation of bio-based products made from sustainable materials that conform to ecological and economic requirements (Narayan, 1994; Narayan, 1998). Synthetic packaging films have led to serious ecological problems due to their non-biodegradability. In this context, biopolymers can be an alternative source for packaging development due to their biodegradablility.

Edible coatings and edible films are two terms used in food packaging, sometimes without any distinction. However, it is important to make such distinction: a "film" is a thin skin formed e.g. through casting of the biopolymer solution prepared separately from the food that is later applied to it, while a coating can be a suspension or an emulsion applied directly on the surface of the food, leading to the subsequent formation of a film. The use of edible films and coatings based on natural polymers and food grade additives has been constantly increasing in the food industry. The coatings/films can be produced with a great variety of products such as polysaccharides, proteins, lipids, resins, with the addition of plasticizers and surfactants.

The functionality and performance of edible coatings/films mainly depends on their barrier and mechanical properties, which in turn depend on film composition, its formation process and the method of application on the product. Edible coatings are gaining importance once they are helping with many challenges related to the storage and marketing of food products, and as an alternative to reduce the deleterious effects imposed by minimal processing e.g. on fresh-cut fruits. The dip coating method is the commonly used method for fruits, cheese, vegetables, fish and meat products. In this method the commodity is directly dipped into the composite coating formulations (in aqueous medium), removed and allowed to air dry, whereby a thin membranous film (coating) is formed over the commodity surface. Continuous dipping builds up decay

organisms, soil and trash in the dipping solution, which needs to be removed for better performance characteristics (Tharanathan and Kittur, 2003). The semipermeable barrier provided by edible coatings is aimed at extending shelf life by reducing moisture and solute migration, gas exchange, respiration and oxidative reaction rates, as well as suppressing physiological disorders on fresh-cut fruits (Baldwin et al., 1996; Park, 1999; Wong et al., 1992). Edible coatings may also serve as carriers of food additives such as antibrowning and antimicrobial agents, colorants, flavors, nutrients and spices (Pranoto et al., 2005).

These edible and biodegradable coatings/films have been successfully utilized in a number of commercial applications: (a) gelatin for capsules, supplements, drugs, and flavor encapsulation; (b) corn zein for coatings, confections, supplements, and drug tablets; (c) collagen for wraps and casings for meat products; (d) starch coatings for drug tablets, confections and dried fruits; (e) methylcellulose, hydroxypropylmethylcellulose, and hydroxymethylcellulose coatings for supplements and drug tablets; (f) fatty acid sucrose esters for coatings for fresh produce; (g) wax, oil, and shellac coatings for fresh produce, confections, supplements, and drug tablets (Krochta, 2002).

2.2 Materials

The basic materials used to produce edible and biodegradable coatings/films in food packaging are the ones directly extracted from biomass. The most commonly available are extracted from marine and agricultural products and they are based in proteins, polysaccharides and lipids.

A schematic diagram of the types of biobased polymers or biopolymers is shown in Figure 2.1. Biobased polymers may be divided into three main categories based on their origin and production (Weber, 2000):

- Category 1: Polymers directly extracted/removed from biomass; examples are polysaccharides such as starch and cellulose and proteins like casein and gluten.

- Category 2: Polymers produced by classical chemical synthesis using renewable biobased monomers; a good example is polylactic acid, a biopolyester polymerised from lactic acid monomers (the monomers themselves may be produced via fermentation of carbohydrate feedstocks).

• Category 3: Polymers produced by microorganisms (either wild strains or genetically modified); to date, this group of biobased polymers consists mainly of polyhydroxyalkonoates (PHA), but developments with bacterial cellulose are in progress.

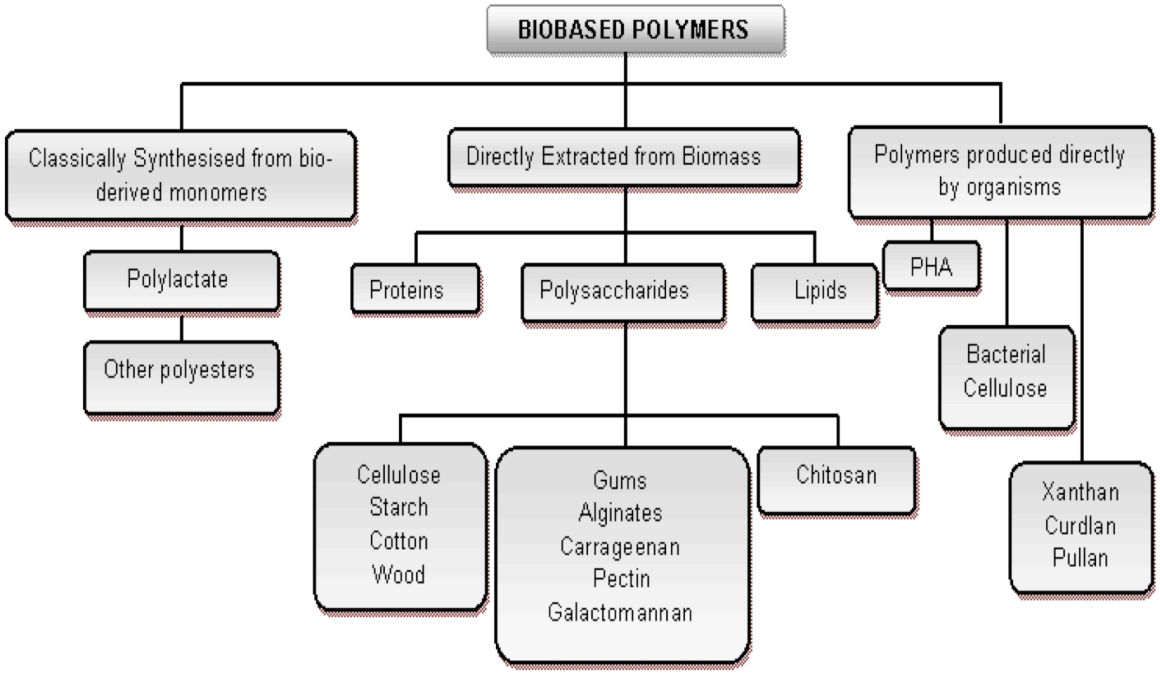

Figure 2.1 - Schematic representation of biobased polymers based on their origin and method of production (Adapted from Weber, 2000).

Polysaccharides are natural polymers that, depending in the source can be neutral, positively or negatively charged. They either act as energy-rich food stores in plants (starch) and animals (glycogen), or have structural roles in the plant cell wall (cellulose, pectin) or the tough outer skeleton of insects and other animals (chitin) (Nelson and Cox, 2000; Nelson, 2000). Polysaccharides that have been evaluated or used to form coatings/films include starch and starch derivatives, cellulose derivatives, alginates, carrageenan, various plant and microbial gums, chitosan, and pectinates (Lin and Zhao, 2007; Rinaudo, 2008). They hydrophilic properties provide a good barrier to CO_2 and O_2 under certain conditions but a poor barrier to water vapor (Guilbert, 1986; Park et al., 1994).

The term "gum" most often specifically denotes a group of industrially useful polysaccharides (glycans) or their derivatives that hydrate in hot or cold water to form viscous solutions or

dispersions at low concentrations (Weber, 2000). When used in foods, gums are sometimes referred as hydrocolloids (Nelson and Cox, 2000), and they are classified as natural and modified (Cuq et al., 1998). Natural gums include seaweed extracts (e.g. alginates), plant exudates (e.g. arabic and tragacanth gums), gums from seeds or roots (e.g. galactomannans and potato starch), and gums obtained by microbial fermentation (e.g. xanthan). Modified gums include mostly cellulose and starch derivatives, such as ethers and esters of cellulose.

2.2.1 Chitosan/Chitin

Chitin is the principal component of the hard exoskeletons of nearly a million species of arthropod insects e.g. lobsters, and crabs; and is probably the second most abundant polysaccharide in nature, next to cellulose. Chitin forms extended fibers similar to those of cellulose, and like cellulose cannot be digested by vertebrates (Nelson and Cox, 2000). By the deacetylation process chitin can be converted into another form of polyaminosaccharide, called chitosan. Chitin is also a common group of solid waste (eg. from films processing industries) in many countries. Utilization of chitosan would provide the additional benefit of minimizing solid waste.

The linear polymer chitin is composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucose (GlcNAc; A-unit) and is insoluble in aqueous solvents. Chitin shares many structural similarities to cellulose, as for example, the conformation of the monomers and the diequatorial glycosidic linkages. Chitosans may be considered as a family of linear binary copolymers of (1→4)-linked A-units and 2-amino-2-deoxy-β-D-glucose (GlcN; D-unit) (Figure 2.2), and do not refer to a uniquely defined compound, but to polysaccharides having different proportions of A- and D-units and of varying chain lengths.

This chemical structure makes chitosan easily modified as immobilization support compared to other materials. Both chitin and chitosan could serve as the material for making carriers for enzymes and cells (Krastanov and Yoshida, 2003; Tartakovsky et al., 1998). Because of their nontoxicity and biocompatibility, the application of chitin and chitosan-based materials covers not only food environmental systems, but also pharmaceutical, medical, and agricultural industries.

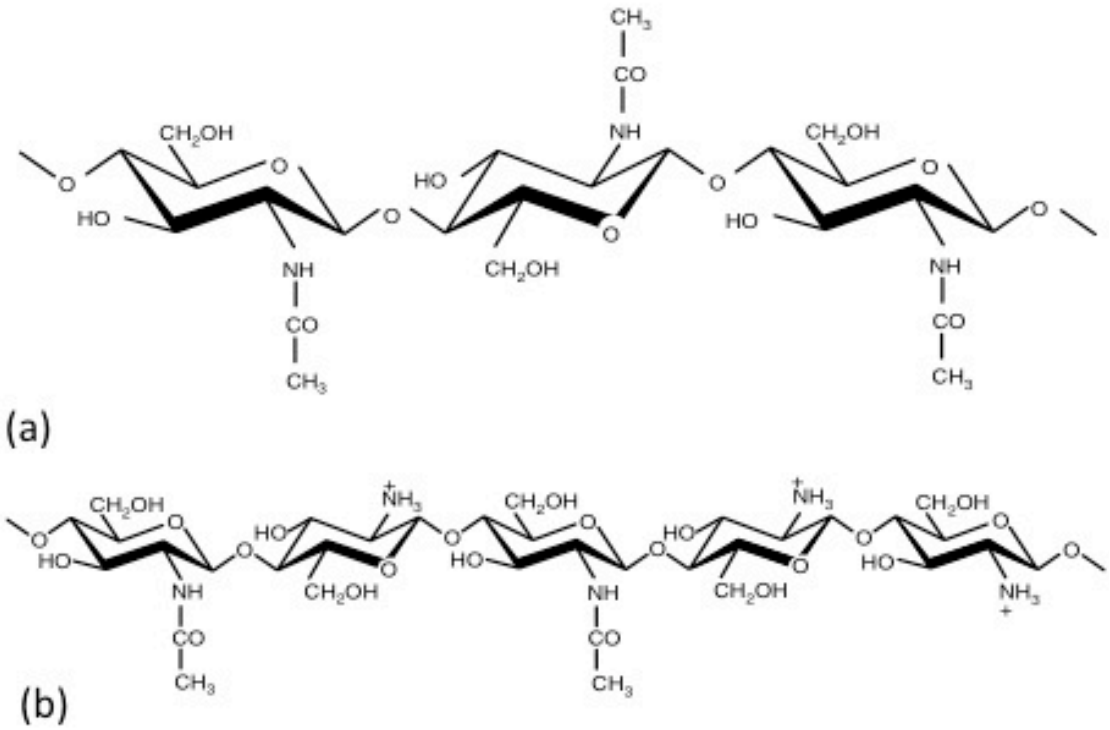

Figure 2.2 - Chemical structure of chitin (a) and chitosan (b).

Chitosan-based materials may be used as edible films or coatings due to their unique property of increased viscosity upon hydration. Furthermore, chitosan films are tough, long-lasting, flexible, and very difficult to tear. Most mechanical properties of chitosan films are comparable to those of many medium-strength commercial polymers (Butler et al., 1996).

The application of chitosans in food for human consumption will involve regulatory approval in the West, although chitin and chitosan could generally be recognized as safe based on their traditional uses in different national food products. Chitin may be found in foods that consist of chitin-containing organisms(Winterowd and Sandford, 1995), such as unpeeled shrimp products and edible fungi containing chitin, including *Aspergillus niger*, *Agaricus campestris* and *Schizophyllum commune* (Winterowd and Sandford, 1995). Chitosans are currently only used in Asia in human food applications, and in the West attempts are underway to obtain generally recognized as safe status from the Food and Drug Administration (FDA) for the use of chitosans as supplements in food (Shahidi et al., 1999). Some potential uses of chitosans in food applications are listed in Table 2.1 (Shahidi et al., 1999).

Table 2.1 - Potential applications of chitin, chitosan, and their derivatives in the food industry

Area of application	Examples
Antimicrobial agent	Bactericidal Fungicidal Measure of mold contamination in agricultural commodities
Edible films	Controlled moisture transfer between food and surrounding environment Controlled release of antioxidants Controlled release of nutrients, flavors, and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning in fruits Reverse osmosis membranes
Additive	Clarification and deacidification in fruits and beverages Natural flavor extender Texture controlling agent Emulsifying agent Food mimetic Thickening and stabilizing agent Color stabilization
Nutritional quality	Dietary fiber Hypocholesterolemic effect Reduction of lipid absorption Production of single cell protein Antigastric agent Infant feed ingredient
Recovery of solid materials	Affinity flocculation
Food processing wastes	Fraction of agar
Purification of water	Recovery of metal ions, pepsticides, phenols Removal of dyes

Adapted from (Shahidi et al., 1999).

2.2.2 Sulfated galactans

Sulfated galactans are among the most abundant non-mammalian sulfated polysaccharides found in nature. They occur in high concentrations, not only in marine red algae (*Rhodophyta*), but are also found in marine invertebrates and in sea grass, a group of vascular plants that occur in the marine environment (Melo et al., 2004). Sulfated galactans from marine algae (also known as carrageenans or agarans) are composed of alternating 3-linked-β-galactopyranose and 4-linked-α-galactopyranose units. However, considerable structural variation occurs among the polysaccharides obtained from different species and collected at different environments or in different periods of the year. A substantial part or even all the α-galactose residues may exist in the form of 3,6-anhydro derivatives. Furthermore, sulfate ester, a methyl group or pyruvic acid may substitute various hydroxyl groups. These structural variations contribute to the highly heterogeneous and complex nature of the sulfated galactans obtained from marine algae (Usov, 1998). Sulfated polysaccharides from algae possess important pharmacological activities such as anticoagulant, antioxidant, antiproliferative, antitumoral, anticomplementary, anti-inflammatory, antiviral, antipeptic and antiadhesive activities (Cumashi et al., 2007; Damonte et al., 2004; de Azevedo et al., 2009). The relationship between structure and biological activities of algal sulfated polysaccharides are not yet clearly established (Bilan et al., 2006). However, it is most likely that some structural features are required for biological activities, especially sulfate clusters to ensure interactions with cationic proteins (Mulloy, 2005). Indeed, the importance of the molecular size has been reported (Albuquerque et al., 2004; Silva et al., 2005).

Structural features of the agar group of polysaccharides are shown in Figure 2.3. They were found to have an agarose-type structure. In general, some C-6 of the 3,6-anhydro-α-L-galactose 3,6-AG) are substituted with sulfate groups resulting in α-L -galactose-6-sulfate, which is known as a biogenetic precursor of 3,6-AG (Rees, 1961).

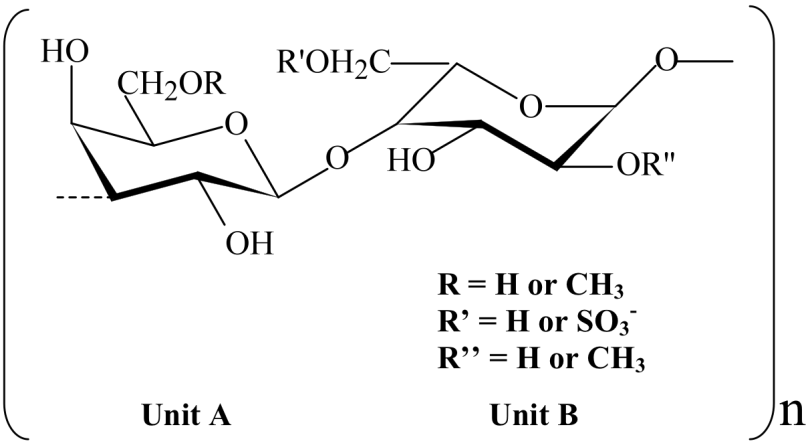

Figure 2.3 - Basic structure of agarans.

Agar is a mixture of polysaccharides found in the cell matrix of red algae (*Rhodophyta*), especially in members from *Gelidiaceae* and *Gracilariaceae* families (Armisen and Galactas, 1987). Agar consists of two different components: agarose and agaropectin. Agarose is a neutral polysaccharide with a linear structure of repeated units of dissaccharide agarobiose, which consists of D-galactose and 3,6-L-galactose. Agaropectin is an acid polysaccharide containing sulfate ester, pyruvic acid and D-glucuronic acid in addition to agarobiose (Araki, 1966). The type and quantity of substituent groups in the polysaccharide chain depends on species (Lahaye and Rochas, 1991), environmental conditions (Bird, 1988), physiological factors (Christiaen et al., 1987; Craigie et al., 1984) and methods of extraction (Freile-Pelegrin and Robledo, 1997; Lemus et al., 1991).

The quality and content of agar depend on its specific physico-chemical characteristics, but are also closely related to environmental parameters (Daugherty and Bird, 1988), growth and reproductive cycle. For example, the agar extracted from two species of *Gracilaria* showed different composition through the seasons (Marinho-Soriano et al., 2001). *G. gracilis* and *G. bursa-pastoris* showed the maximum yield during spring (30 %) and summer (36 %), while the minimum was observed during autumn (19 %) and winter (23 %), respectively (Marinho-Soriano and Bourret, 2003). In addition, the gelling temperature showed significant seasonal variation for both species and, in general, the agar extracted from *G. gracilis* possessed better qualities than agar extracted from *G. bursa-pastoris* and can be considered a candidate for industrial use (Marinho-Soriano and Bourret, 2003).

The yield and physical properties of agar, such as gel strength and gelling temperature as well as

chemical properties, determine its commercial value (Marinho-Soriano and Bourret, 2005). Low quality agar is used in food products (frozen foods, bakery icings, meringues, dessert gels, candies and fruit juices). Industrial applications include paper sizing/coating, adhesives, textile printing/dyeing, castings, impressions, etc. Medium quality agars are used as the gel substrate in biological culture media and are also important in the medical/pharmaceutical field as bulking agents, laxatives, suppositories, capsules, tablets and anticoagulants. The most highly purified and upper market types (the neutral fractions called agarose) are used for separation in molecular biology applications (electrophoresis, immunodiffusion and gel chromatography).

Although little commercial exploitation of agars occurs outside the hydrocolloid industry, they have been recently employed in medicinal and pharmaceutical areas such as in a therapy against cancer cells since it can induce the apoptosis of these cells *in vitro* (Cardozo et al., 2007).

Nowadays the application and studies of agar film have been may be due to the fact that these polysaccharides have particular characteristics: their films are clear and strong, but are insoluble in water under ambient conditions (Phan the et al., 2009).

Phan et al., (2009) studied the potential use of agar as edible packaging and its functional properties; these authors observed that the moisture barrier properties of edible films made of agar are comparable to those of other polysaccharides and their derivatives or some protein films. Agar-based films display a higher moisture sorption at the same RH conditions and they seem to have better moisture barrier properties than other films. Due to their relatively high Water vapor permeability (*WVP*) values and their hygroscopicity, the potential use of these films as moisture barrier packaging or coating is restricted. However, some polysaccharides, when used in the form of a high-moisture gelatinous coating, will retard water loss from some foods during short-term storage; the above studied films could be used in the same way.

Especially agar films, plasticized with glycerin, are transparent, clear, homogeneous, flexible, and easily handled. Such mechanical properties give a promising utility for their use as packaging or coating because they can preserve not only the integrity of the products but also other functional properties of the films themselves.

2.3 Functional properties of edible films and coatings

The potential of edible films to control water transfer, and to improve food quality and shelf life, has received increasing attention from researchers and industry (Carneiro-da-Cunha et al., 2009; Casariego et al., 2008; Cerqueira et al., 2009; Lima et al., 2010). An edible coating or film has been defined as a thin, continuous layer of edible material formed or placed on or between foods or food components (Carneiro-da-Cunha et al., 2009). The aim is to produce natural biopolymer-based coatings materials with specific properties, which may be eaten together with the food (Bravin et al., 2006). A great diversity of materials is used to produce edible coatings and films. They are extracted from marine and agricultural animals and plants e.g. lipids, polysaccharides and proteins. Edible coatings formulated with the desired properties can be utilized by the food industry to meet challenges associated with long term quality, market safety, nutritional value and economic production cost. With regard to the fresh products industry, the potential benefits of using edible coatings include (Figure 2.4):

1. Providing a moisture barrier on the surface of the product thus minimizing the problem of moisture loss. Moisture loss during postharvest storage of fresh products leads to weight loss and changes in texture, flavor and appearance;
2. Providing sufficient gas barriers for controlling gas exchange between the fresh product and its surrounding atmosphere, slowing down respiration and delaying deterioration. The gas-barrier function could in turn retard the enzymatic oxidation and protect the fresh product from browning discoloration and texture softening during storage;
3. Restricting the exchange of volatile compounds between the fresh product and its surrounding environment, again by providing gas barriers, which prevents both the loss of natural volatile flavor compounds and color components from fresh product and the acquisition of different odors;
4. Protecting the product from physical damage caused by mechanical impact, pressure, vibrations and other mechanical factors;
5. Acting as carriers of functional ingredients, such as antimicrobial and antioxidant agents, nutraceuticals, and color and flavor ingredients for reduction of microbial loads, delaying oxidation and discoloration, and improving the overall quality (Rooney, 2005).

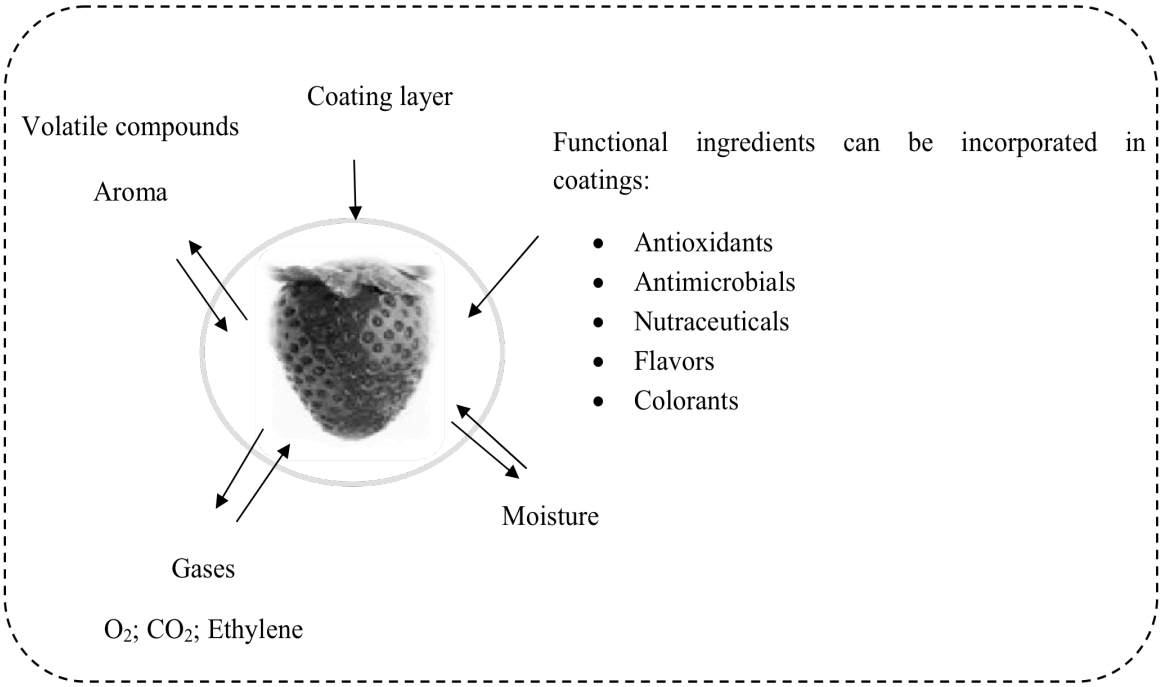

Figure 2.4 - Functional properties of an edible coating on fresh fruits and vegetables. Adapted from Lin and Zhao (2007).

2.4 Surface properties

The effectiveness of edible coatings depends primarily on the control of the wettability (*Ws*) of the coating solutions (Park, 1999). They must wet and spread on the surface of the food product, and upon drying they must form a film that has the adequate properties and durability. The coating process involves wetting of the food product by the coating solution, and the possible penetration of the solution into the skin (Hershko et al., 1996). Also the surface energy or surface tension of the food product is a controlling factor in the process that involves wetting and coating of surfaces (Karbowiak et al., 2006). The determination of surface tension usually involves measuring the contact angles that several standard liquids make with that surface. The surface energy of the solid surface is then related to the surface tensions of the liquids and the contact angles. This method invokes an estimation of the critical surface tension of the surface of the solids studied, by extrapolation from the Zisman plot (Zisman, 1964).

The wettability is obtained by determining the values of the spreading coefficient (*Ws*) and the works of adhesion (*Wa*) and cohesion (*Wc*). The surface tension of the coating solution is measured by the pendant drop method using the Laplace-Young approximation (Guilbert, 1986). The contact angle (θ) of a liquid drop on a solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions: solid-vapor (γ_{SV}), solid-liquid (γ_{SL}), and liquid-vapor (γ_{LV}) (Figure 2.5). The equilibrium spreading coefficient (*Ws*) is defined by Equation 2.1 (Rulon and Robert, 1993) and can only be negative or zero.

$$Ws = Wa - Wc = \gamma_{sv} - \gamma_{Lv} - \gamma_{SL} \quad \text{Eq 2.1}$$

Where *Wa* and *Wc* are the works of adhesion and cohesion, defined by Equation 2.2 and Equation 2.3, respectively.

$$Wa = \gamma_{LV} + \gamma_{SV} - \gamma_{SL} \quad \text{Eq 2.2}$$

$$Wc = 2 \cdot \gamma_{LV} \quad \text{Eq 2.3}$$

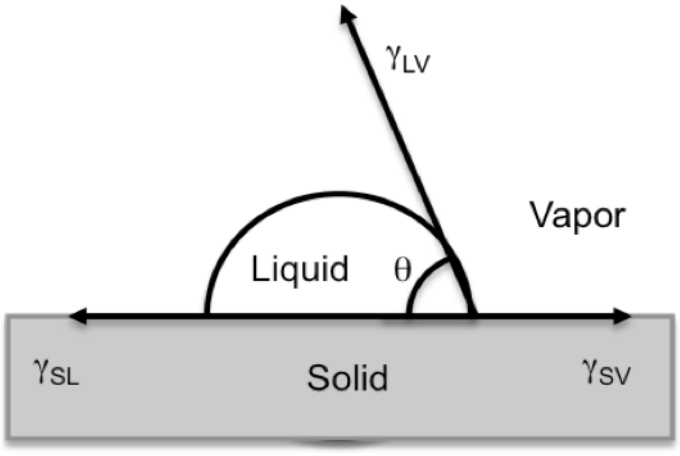

Figure 2.5 - Schematic of a sessile drop, contact angle (θ); the three interfacial tensions (γ_{LV}: liquid–vapor, γ_{SV}: solid–vapor, and γ_{SL}: solid–liquid) are shown.

Choi et al. (2002) studied the wettability of chitosan coating solutions on 'Fuji' apple skin using the Du Nouy ring method and the sessile-drop method. The 'Fuji' apple skin surface presents a critical tension of 18.7 $mN \cdot m^{-1}$. Ribeiro et al. (2007) obtained a similar value for the critical surface tension of strawberries (18.84 $mN \cdot m^{-1}$), which were reported to have a superficial tension of 28.94 $mN \cdot m^{-1}$, with polar and dispersive components of 5.95 and 22.99 $mN \cdot m^{-1}$, respectively. In both cases, to enhance the wettability of the coating solutions, Tween 80 was added, reducing the superficial tension of the liquid and thus increasing the spreading coefficient.

Casariego et al. (2008) determined the effects of the concentrations of plasticizers, Tween 80 and chitosan on the wettability of chitosan-based edible coatings in view of their application on tomato and carrot. They present the superficial tensions of tomato and carrot as being 28.71 and 26.48 $mN \cdot m^{-1}$, respectively. The increase of chitosan concentration and the presence of glycerol or sorbitol as plasticizers decreased the values of wettability and adhesion coefficients. The best experimental values of wettability were obtained for the coating composition of 1.5 % (w/v) of chitosan and 0.1 % (w/w) of Tween 80.

A good choice of the coating formulation is essential for the durability and maintenance of the coating on the food products. The determination of wettability, with the study of the spreading coefficient, work of adhesion and cohesion, as well as the study of the surface properties of the products, is therefore fundamental for the correct application of edible coatings. Further work has to be performed to understand how different factors as such temperature and the application method can be important in the coating performance (Guilbert, 1986; Guilbert et al., 1997).

2.5 Film forming mechanisms

The film forming mechanisms of biopolymers include intermolecular forces such as covalent bonds (e.g., disulfide bonds and cross-linking) and/or electrostatic, hydrophobic or ionic interactions. For the resulting films or coatings to be edible, the film forming mechanism involved in fabrication should be an appropriate food process, namely pH modification, salt addition, heating, enzymatic modification, drying, use of food-grade solvents and addition of other food-grade chemicals. The control of fabrication process conditions is very important because changes in treatment conditions can alter kinetics and reaction mechanisms.

Han and Gennadios (2005) described potential chemical and physical approaches to the modification of film forming mechanisms by altering film forming raw materials, varying film forming processing conditions, and applying treatments on formed films (Figure 2.6). As examples, potential chemical methods for modifying the film forming mechanisms of protein-based films include pH changes, salt addition, heat denaturation, solvent changes, chemical modification of the side chains of peptides, cross-linking, and hydrolysis of peptides (Rhim, 1998; Were et al., 1999; Yildirim and Hettiarachchy, 1998), irradiation of peptides (Lacroix and Ouattara, 2000), and the addition of foreign proteins. For polysaccharide-based films several chemical modifications are available, including salt addition, solvent changes, heat gelatinization, pH changes, chemical modification of hydroxyl groups, cross-linking of polysaccharides, hydrolysis of polysaccharides, and the addition of foreign polysaccharides (Conca, 2002). Physical modifications of edible films and coatings include lamination, formation of composites, addition of particles or emulsions, perforation, over-coating, annealing heat curing, orientation, radiation, ultrasound treatment, and electric field treatments Mechanical or physicochemical properties of films can be improved by application of extrusion or promotion of reaction between film components (Banejee et al., 1996).

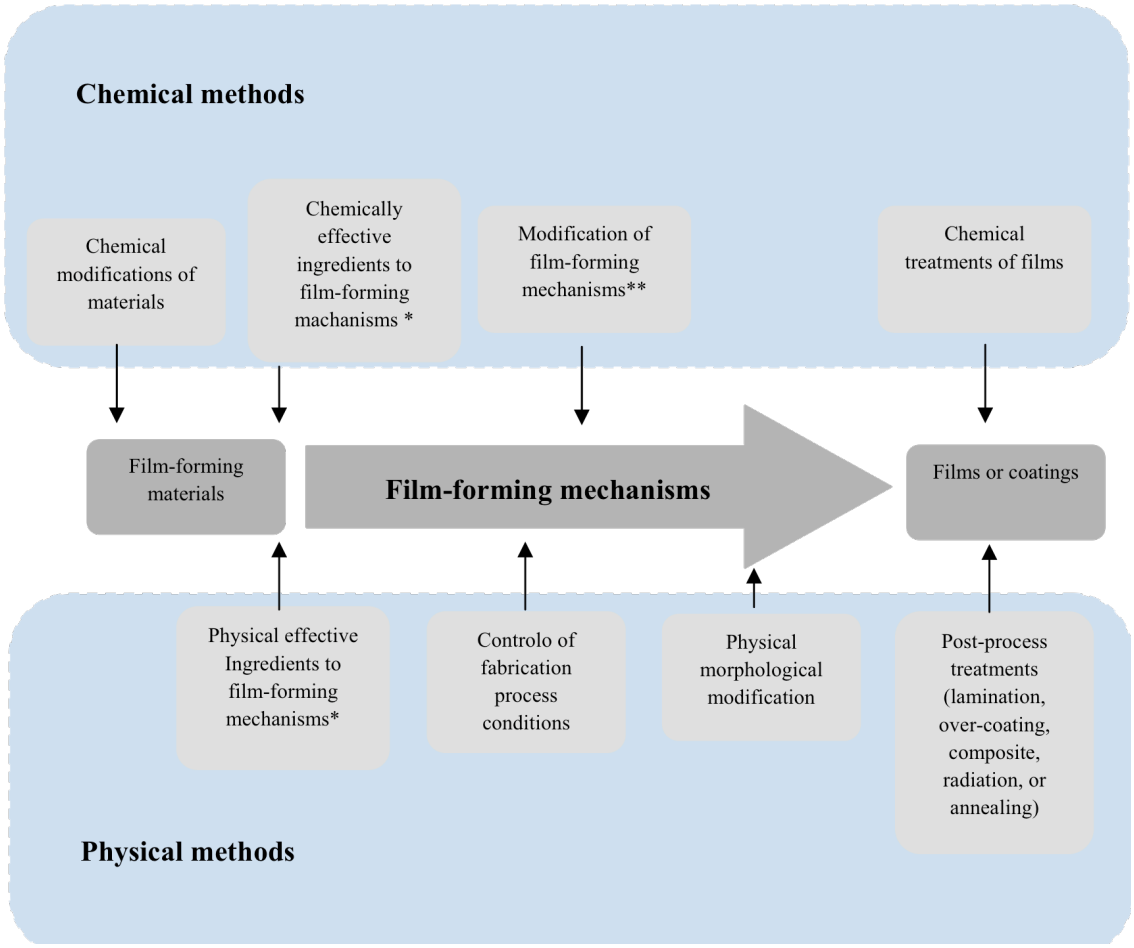

Figure 2.6 -Various ways of modifying the characteristics of edible films and coatings.

*Indicates the addition of chemically or physically active ingredients, which may enhance or interfere with the film forming mechanisms; ** includes any chemical cross-linking, chemical substitution of side chains to create hydrophobic interaction or electrostatic interactions, and other extra mechanisms caused by chemical modifications. Adapted from (Lacroix, 2009).

2.6 Application of ohmic heating and electric fields

Currently, technologies utilizing electrical energy directly into food processing raised a significant interest in the food industry. Several of those technologies are now being used on a commercial scale for processing of an extensive range of food products. Research in this area will provide the food processor the opportunity to produce new and value-added food products with enhanced

quality attributes preferred by consumers (Han and Gennadios, 2005; Manvell, 1997; Sastry and Barach, 2000; Zareifard et al., 2003).

Ohmic heating is based on the passage of electrical current through a food product that has electrical resistance. The electrical energy is converted to heat. Instant heating occurs depending on the current passing through the food material. One possible device to apply ohmic heating is illustrated in Figure 2.7. For this type of systems the electric field strength can be varied by adjusting the electrode gap or the applied voltage; one of the most important factors is the electrical conductivity of the product and its temperature dependence (Ruan et al., 2004). If the product consists of more than one phase such as in the case of a mixture of liquid and particulates, the electrical conductivity of all the phases has to be considered (Ruan et al., 2004). The electrical conductivity increases with rising temperature for most food materials, suggesting that ohmic heating becomes more effective as temperature increases, but this could result in runaway heating (Ruan et al., 2004). Differences in the electrical resistance and the temperature dependence between the two phases can complicate the heating characteristics of the system. Electrical conductivity is influenced by the ionic content being possible to adjust the electrical conductivity of a product by adding electrolytes (e.g. salts) to manipulate the heating patterns and improve the effectiveness of ohmic heating.

Ruan et al. (2004) reported that the influence of ohmic heating on food occurred when an electrical current passed through it, resulting in the temperature rise in the products due to the conversion of electric energy into heat (Joule effect).

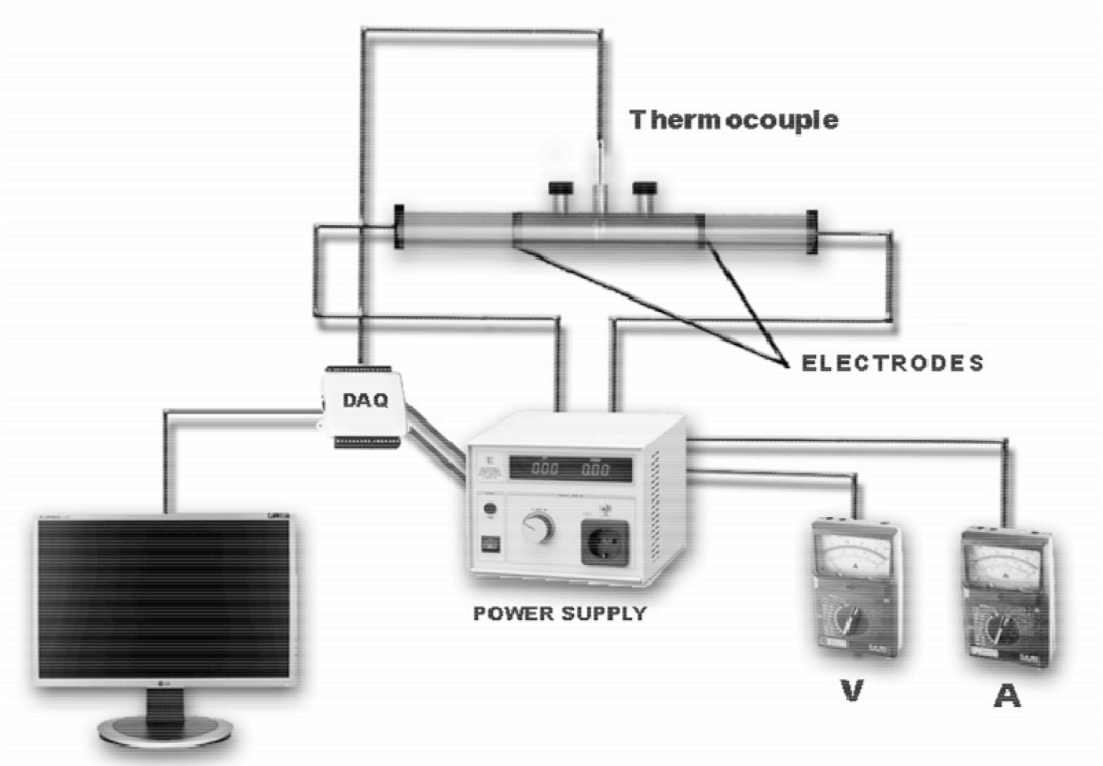

Figure 2.7 - Schematic diagram showing the device used to perform ohmic heating. DAQ = Data Acquisition. V= Voltage. A= Amperage.

Ohmic heating of food products is used in a wide range of applications such as preheating, blanching, pasteurization, sterilization and extraction. Its advantages compared to conventional heating include maintaining the color and nutritional value of food, short processing time and higher yield (Castro et al., 2004; Icier and Ilicali, 2005; Ruan et al., 2004; Vikram, 2005).

Ohmic heating technology has gained importance because the products are of a superior quality to those processed by conventional technologies (Castro et al., 2003; Kim et al., 2002; Wang, 2002). Moreover, the ohmic heater assembly can be incorporated into a complete product sterilization or cooking process. Among the advantages claimed for this technology are uniformity of heating and improvements in quality, with minimal structural, nutritional or organoleptic changes (Parrott, 1992).

The application of electric fields has also been addressed by researchers in the area of edible films and coatings, and there are works showing that the application of electric fields promotes a

significant improvement of several properties . Results show that the application of electric fields to chitosan film forming solutions may be an important instrument to tailor films' properties. Until now only two methods have been tested, which are summarized below: 1) the application of the electric field to the film forming solutions with subsequent drying; (Skuder, 1989) and 2) the application of the electric field during the drying process .

García et al., (2009b), studied the effects of applying an electric field on films during their drying process. The electric field was applied parallel to the surface of the film in order to obtain axially oriented films. The electrically treated films were obtained by casting in a house-built cell with a positive and negative electrode, placed inside an oven at 60 °C. The electrodes were set into acrylic plates filled with different film forming solutions and connected to a 12 V power supply. Control films were obtained by casting, and dried at 60 °C in an oven until constant weight. The authors showed that, electrical field treatment could be a good alternative to improve film flexibility and to increase water vapor barrier properties.

2.6.1 Influence of electric fields in edible coatings/films properties

Edible films and coatings are designed to function as a barrier to moisture, water vapor, gas (O_2 and CO_2), flavor and aroma, thus improving food quality and increasing its shelf life (Souza et al., 2009).

Both O_2 and CO_2 permeability is important when respiration or oxidation reactions could affect the quality of the food. Mechanical properties of edible coatings/films are important in food protection, reducing bruising and split, protecting food integrity. Film solubility is also an important property: in some cases, a water-insoluble film or coating is preferred in order to provide water resistance and improve food integrity (García et al., 2009b; Krochta, 2002); in other cases, edible films with high water solubility may be required (Lacroix, 2009).

2.6.2 Structure characterization

Scanning electronic microscopy *(SEM)* and X-ray diffraction (*XRD*) are frequently used to characterize edible films' structure (Guilbert, 1996; Sébastien et al., 2006). *SEM* may be used to evaluate film homogeneity, layer structure, pores and cracks, surface smoothness and thickness while *XRD* is mainly used to evaluate the degree of cristallinity of the films.

García et al., (2009b), showed that when chitosan film forming solutions were submitted to an electric field, chitosan films presented crystals in their structure, evidencing the occurrence of morphological influences from the treatment. *SEM* analyses indicated that the electric treatment significantly modified the structure of the films towards a more regular structure, which may presumably be reflected on the changes observed in the surface morphology of the film.

Crystallinity measures the extent of organization of the molecules in a polymer. Polymer properties that affect crystallinity include the structural regularity of the polymer chains; polymer chain mobility, which allows variable conformation; the repeating presence of side chains, which engage in intermolecular bonding; and the absence of bulky side chains, which interfere with the crystal lattice formation (Souza et al., 2010).

The mass transfer of gas in a semi-crystalline polymer is primarily a function of the amorphous phase, because the crystalline phase is usually assumed to be impermeable. As the percent crystallinity of a polymer increases, the oxygen permeability decreases. The degree to which oxygen permeability is affected is highly dependent on polymer structure (García et al., 2009b).

Usually, polymers possess a high degree of crystallinity, forming an organized structure that has good mechanical properties (Miller and Krochta, 1997). *XRD* can be used to track recrystallization of film polymers during storage. In the case of starch-based films, peak width decreased slightly and peak intensities increased, indicating a growth in crystallite size corresponding to a slow recrystallization process. *XRD* patterns of composite films generally represent a mixture of component features in which the characteristic peaks of individual components can be identified (Miller and Krochta, 1997).

Balau et al. (2004) studied the X-ray difractograms of chitosan films, an almost amorphous structure. They showed that films treated with an electric field of E = 20 kV cm^{-1}, developed a crystalline structure, while the films to which no electric field was applied displayed a significantly lower proportion of crystalline material, showing that the electric field plays an important role in the crystallization process.

Garcia et al. (2009b) indicated that, during film drying under an electric field, a sharp peak at 2θ = 15° appeared and developed a structure with a different X-ray diffraction pattern. This structure was more ordered, since the crystallinity index *(CI)* of chitosan treated film was of 14 % when comparing to the value for untreated films).

2.6.3 Transport properties

Permeability is a steady-state property that describes the extent to which a permeating substance dissolves and then the rate at which it diffuses through a film, with a driving force related to the difference in concentration of that substance between the two sides of the film (García, 2009b). Gas permeabilities of edible films and coatings depend on several factors such as the integrity of the film, the ratio between crystalline and amorphous zones, the hydrophilic-hydrophobic ratio and the polymeric chain mobility; the interaction between the film forming polymer and the presence of a plasticizer or other additives are also important factors in film permeability (Souza et al., 2010).

Oxygen is the key factor that might cause food oxidation, inducing several unwanted changes such as in odor, color and flavor, as well as nutrients deterioration. Therefore, films providing a proper oxygen barrier can help improving food quality and extending its shelf life (Conca, 2002).

Carbon dioxide is formed in some foods due to deterioration and respiration reactions. The CO_2 produced has to be removed from the package to avoid food deterioration and/or package destruction (Garcia et al., 2000).

The determination of CO_2 and O_2 transport properties is very important because foods may suffer biological, chemical and physical deterioration during storage and distribution; this is specially the case of fruits and vegetables.

Caner et al. (1998) showed how the gas permeation properties of chitosan films were affected by acid and plasticizer concentrations. They showed that higher concentrations of plasticizer (PEG 400) lead to higher values of O_2P explaining this behaviour by the plasticizer effect, that decreases the intermolecular attractions between polymeric chains, facilitating the penetration of gas molecules. Also the type of acids used in film formulation effect the O_2P, being the lactic acid the one providing the lowest values, when compared with acetic and propionic acid. More recently, Srinivasa et al. (2007) studied the O_2P of chitosan films with different plasticizers (glycerol, sorbitol and PEG) and fatty acids (stearic and palmitic acids). In that work, the addition of glycerol and sorbitol into chitosan films increased O_2P, whereas the addition of PEG decreased the O_2P of chitosan films when compared with chitosan films without plasticizer. Also Cerqueira et al. (2009b) showed that the increase of the plasticizer concentration (glycerol) decreased the O_2P and CO_2P in chitosan films.

Garcia et al. (2000) studied how the permeability properties of starch based films could be affected by the presence of lipids, the type of starch and the plasticizer. They showed that the absence of plasticizer in starch films leads to an increase of O_2P and CO_2P, attributed to the presence of cracks and pores in the unplasticized films. The presence of the lipid did not affect the CO_2P, and the films with sorbitol presented the lowest values of O_2P and CO_2P.

Water vapor permeability (*WVP*) is an important parameter commonly considered in food packaging. The measured *WVP* of the films is determined as follows:

$$WVP = (WVTR.L) / \Delta P \qquad \text{Eq 2.5}$$

Where *WVTR* is the measured water vapor transmission rate ($g \cdot m^{-2} \cdot s^{-1}$) through the film (calculated from the slope of the curve divided by the area of the film), *L* is the mean film thickness (m), and ΔP is the partial water vapor pressure difference (Pa) across the two sides of the film. *WVP* comprises sorption, diffusion and adsorption and is largely governed by the interactions between the polymer and water molecules (Vermeiren et al., 2003). Water permeation through a film usually occurs through the hydrophilic part of the film, thus the relation of the hydrophilic/hydrophobic portions is important on *WVP*. Polymers with high hydrogen bonding produce films that are susceptible to moisture while polymers with hydrophobic groups make excellent barriers to moisture.

Generally, *WVP* is also dependant on the pore size of the film (Paramawati et al., 2003). In fact, *WVP* tends to increase with polarity, degree of unsaturation and degree of ramification of the lipids used (if any), in addition to the effect of water molecules sorption by the polar part of the film material (Nivedita, 2004). Nevertheless, the poor water vapor barrier may provide some benefit since it allows movement of water vapor across the film, thus preventing water condensation, which is a potential source of microbial spoilage (Nivedita, 2004). The water vapor permeability should be as low as possible since an edible film or coating should retard moisture transfer between the food and the environment, or between two components of a heterogeneous food product (Gontard, 1994). (Table 2.2) shows some *WVP* values those of other hydrocolloid-based films reported in the literature (Balau et al., 2004; Bravin, 2006; Du et al., 2007; Krokida, 2003). The work of Miller and Krochta, (1997) points at the fact that the permeability is highly

affected by how closely packed the polymer chains are, thus establishing a direct relationship between the crystallinity of the structure and permeability.

Table 2.2 - Comparison of *WVP* values of edible films

Films composition	WVP ($gm^{-1}s^{-1}Pa^{-1}$)	References
Alginate-calcium: PEG 400	≈ 65 x 10^{-11}	(Miller and Krochta, 1997)
Chitosan (96% degree acetylation):glycerol	30.6 x 10^{-11}	(Krokida, 2003)
Chitosan (96% degree acetylation)	24.7x 10^{-11}	(Krokida, 2003)
Starch/chitosan:glycerol	39.2 x 10^{-11}	(Balau et al., 2004)
Starch/chitosan:glycerol: ferulic acid	31.9x 10^{-11}	(Balau et al., 2004)
Starch:MC	20.5 x 10^{-11}	(Du et al., 2007)
Starch:MC:soyben oil	11.7 x 10^{-11}	(Du et al., 2007)
Chitosan:Tween 80	≈ 160 x10^{-11}	(Bravin, 2006)
Chitosan:Tween 80: oleic acid	≈ 78 x 10^{-11}	(Ziani et al., 2008)

2.6.4 Solubility in water

Solubility in water is defined as the maximum percentage (by weight) of a substance that will dissolve in a unit volume of water at certain (usually room) temperature. It is an important property, which governs potential applications of these materials to food preservation.

The results observed by (Caner et al., 1998), showed that methylcellulose films were completely soluble in water while control chitosan films had lower solubility values. Composite samples had intermediate water solubilities, which decreased with increasing chitosan proportion. In all cases electrically treated film samples resulted less soluble than control samples, regardless of chitosan content.

García et al. (2009b) showed that the electric field plays an important role in the crystallization process, which may also interfere in the water solubility of the films.

Lei et al. (2007) showed that during the rehydration process, the amount of water absorbed had a quick increase during the first 10 min, when a maximum was reached, for both heating methods (ohmic and water bath heating). However the maximum amount of water absorbed by the protein-lipid film was larger for the film formed by ohmic heating than for the film formed by water bath heating, meaning that the rehydration rate was higher in the first case. This change was probably caused by a different degree of cellular and structural destruction of the protein-lipid film, once it has been described that the degree of rehydration is dependent on the degree of cellular and structural disruption (Lei et al., 2007).

2.6.5 Mechanical properties

Tensile strength *(TS)* and elongation-at-break *(E)* are frequently used to characterize the mechanical properties of films. *TS* is the maximum tension supported by the film until the moment it collapses. *E* is a measure of the flexibility of the film and can be considered as a characteristic that defines the ability of the film to deform in place before it collapses. *TS* is expressed in MPa and is calculated by dividing the maximum load (N) by the cross-sectional area (m^2). Percent elongation *(E)* at break is determined by dividing the length of the samples at the moment of rupture by the initial gauge length of the samples and multiplying by 100. These measurements are important once the mechanical properties of films or coatings depend on the filmogenic nature of the material used, which is directly related to its structural cohesion (Lacroix et al., 2009). Multiple factors, such as film composition, temperature, relative humidity, and storage time, affect tensile properties. Generally, the addition of plasticizers leads to a decrease of *TS* and an increase of *E* (Grashchenkov et al., 1959; Lacroix et al., 2009).

Park et al. (2002) refer that the increase of the values of *TS* and *E* for chitosan films is related with the deacetylation degree of the sample.

Chen et al. (1994) and Ziani et al. (2008) mentioned that those results were due to the higher crystallinity of chitosan films. The polymer chain of chitosan with a higher degree of deacetylation was reported to be more mobile, and this increased mobility was in turn related with an easier formation of inter- or intra-chain hydrogen bonds. This leads to a higher crystallinity that reduces the absorption of water molecules and produces an increase of *TS* (Cervera et al., 2004). In

general, the crystallinity of films is directly related with increased intermolecular forces, thus increasing the rigidity and brittleness of the film (Ziani et al., 2008).

Garcia et al. (2009) showed that electrically treated samples exhibited higher *E* values, indicating that the electric treatment allowed the alignment of the chains in the field direction, facilitating their stretching and thus increasing their flexibility.

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Chapter 3 - Chemical characterization and antioxidant activity of sulfated polysaccharide from the red seaweed *Gracilaria birdiae*

3.1 Introduction

In recent years, marine resources have attracted attention in the search for bioactive compounds to develop new drugs and healthy foods (Qi et al., 2005). In particular, seaweeds are a very important and commercially valuable resource for food, fodder, soil conditioners and pharmaceuticals (Yang et al., 2006). Moreover, sulfated polysaccharides from marine algae are known to exhibit many biological and physiological activities including anticoagulant, antiviral, antitumor, anti-inflammatory and antioxidant (Becker et al., 2007; F-Tischer et al., 2006; Souza et al., 2007; Ye et al., 2008).

Red seaweeds synthesize a great variety of sulfated galactans, which are the major components of the extracellular matrix. Polysaccharides from the *Gracilaria* genus are composed mainly of the alternating 3-linked-β-D-galactopyranose unit (Gal) and the 4-linked-3,6-anhydro-α-L-galactopyranose unit (AnGal). The Gal unit can be substituted by either a methyl or a sulfate ester groups (Rees, 1961).

Marine red algae of the genus *Gracilaria* are a major agarophyte resource in the world and are cultivated for the phytocolloid industry or for integrated marine culture (Troell et al., 2003). The red marine alga *Gracilaria birdiae* has a great economic impact in Brazil due to agar production (Plastino et al., 2004). Marinho-Soriano, (2001) studied the cultivation of this species in the sea and (Maciel et al. (2008) studied the isolation and structural characterization of the cold water-soluble fraction of polysaccharide taken from *G. birdiae* cultivated on the Atlantic coast of Brazil (Fleixeiras Beach, in the State of Ceará). No studies have yet been performed on the chemical characterization of its polysaccharides extracted with hot water.

When the natural defenses of an organism (of enzymatic, non-enzymatic, or dietary origin) are overwhelmed by an excessive generation of reactive oxygen species (Lloyd et al., 1961), a situation of 'oxidative stress' occurs. Consequently, cellular and extracellular macromolecules (proteins, lipids, and nucleic acids) can suffer oxidative damage, causing tissue injury (Halliwell and Auroma, 1991; Halliwell and Gutteridge, 1989). Most organisms are able to defend themselves from oxidations and repair oxidative damages. However, the innate defense in the human body may not be enough for severe oxidative stress. Antioxidants are substances that can delay or prevent oxidation of cellular oxidizable substrates (Wang et al., 2009). Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the amount of ROS (Li, 2007).

In order to reduce damage to the human body and prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. The most commonly used antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) (Qi et al., 2005).

In recent years, algal polysaccharides have been demonstrated to play an important role as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms (Kim et al., 2007; Souza et al., 2007; Wang et al., 2009). More recently Cerqueira et al. (2009) studied the applicability of sulfated polysaccharide from *G. birdiae* as cheese coatings. Polysaccharides from seaweeds have also interesting thickening and gelling properties. Since the rheological behaviour is directly linked to the structure of the polysaccharide, the rheological characterization of sulfated polysaccharide from *G. birdiae* is required.

The aim of this work was to obtain sulfated polysaccharides from *Gracilaria birdiae* isolated by aqueous extraction at 90 °C and evaluate their chemical and rheological properties and to test their antioxidant potential.

3.2 Materials and methods

3.2.1 Chemical analyses

Total sugar content of each fraction was determined according to the method of Dubois et al. (1956). Protein content was measured by Bradford's method (Bradford, 1976).

3.2.2 Extraction of polysaccharides

Specimens of the red seaweed *G. birdiae* were collected in the Atlantic coast of Brazil (Fleixeiras Beach, Trairi, Ceará). This species grows attached to rocks or dead coral. The diploid phase that develops directly on the female thallus, the carposporophyte, is evident all year in the area, and was selected as seed material. The seedlings were cleaned and then tied in a structure made of string, which was placed in the sea (03° 13´ 25" S and 039° 16´ 65" W), where it was anchored and submerged for

two months. After that period algae were collected, cleaned of epiphytes, washed with distilled water and stored at - 20 °C.

The samples were air dried and then milled. The powder was extracted with water (1.5 % w/v) at 25 °C with mechanical stirring for 15 h. The algal residue was removed by filtration and supernatant discarded. The algal residue obtained from the first extraction was extracted with water at 90 °C with mechanical stirring for 45 min. The residue was removed by centrifugation, and the supernatant was precipitated with ethanol (1:3 v/v), lyophilized and stored.

3.2.3 Determination of sulfate content in polysaccharides

Sulfate content in polysaccharides was determined by the barium chloride–gelatin method (Lloyd et al., 1961) . A standard curve was made as follows: 0.02; 0.04; 0.06; 0.08; 0.10; 0.12; 0.16; 0.18 and 0.20 mL K_2SO_4 standard solution (0.6 mg mL^{-1}) were accurately put into test tubes; hydrochloric acid (1M) was compensated to 0.2 mL solution. Then 3.8 mL of trichloroacetic acid (3 % v/v) and 1.0 mL of barium chloride-gelatin solution (5 g L^{-1}) were added, vortexed, and absorbances were measured at 360 nm after incubation for 15 min at room temperature; 0.2 mL hydrochloric acid solution was used as a blank.

3.2.4 Polysaccharide analyses

The monosaccharide composition of red seaweed galactans was obtained by reductive hydrolysis (Stevenson and Furneaux, 1991). This technique combines hydrolysis and reduction followed by acetylation to produce alditol acetates. After acetylation, these alditol acetate derivatives were analyzed by gas chromatography (GC), (Carlo Erba 6000, Carlo Erba, Milan, Italy) with a split injector (split ratio 1:60) and a flame ionization detector. The column was a DB-225 (J & W, USA) with 30 m x 0.25 mm and film thickness of 0.25 μm; the oven temperature program was: 220 °C during 5 min, being then the temperature raised at a rate of 20 °C min^{-1} to 230 °C and maintained at this temperature for further 6 min. The flow rate of the carrier gas (H_2) was set at 1 mL/min at 220 °C. The injector temperature was 220 °C and the flame ionization detector temperature was 230 °C. The hydrolysis of all samples was performed in duplicate and each one was injected twice.

3.2.5 Molar mass distribution

The peak molar masses (M_{pk}) were estimated by gel permeation chromatography (GPC) with a Shimadzu equipment at room temperature using an Ultrahydrogel linear column (7.8 X 300 mm, exclusion limits 10^6 g mol^{-1}), flow 0.5 mL min^{-1}, 0.5 % polysaccharide concentration and 0.1 M $NaNO_3$ as solvent. A differential refractometer and an ultraviolet photometer (at 280 nm) were used as detectors and the elution volume corrected to the internal marker of ethylene glycol at 10.45 mL. Pullulan samples (Shodex Denko) of M_w 5.9 x 10^3, 1.18 x 10^4, 4.73 x 10^4, 2.12 x 10^5 and 7.88 x 10^5 g mol^{-1} were used as standards.

3.2.6 FTIR analyses

The IR spectra of the polysaccharides were determined using a Fourier transform infrared spectrometer (FTIR) (Perkin-Elmer 16 PC spectrometer, Boston, USA). The polysaccharide was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FTIR measurement in the wavenumber range of 600 and 4000 cm^{-1} using 16 scans.

3.2.7 Rheological measurements

The measurements under frequency sweep of *G. birdiae* sulfated polysaccharide and commercial-grade agar (Fluka, Switzerland) have been performed using a controlled strain rheometer (AR-G2) from TA Instruments (USA), using a plate and plate geometry (diameter: 25 mm) with a gap between plates of 1 mm. Frequency sweeps were performed, at 25 °C, in the 0.1–10 Hz range and the strain was fixed at 0.7 % in order to assure that the working conditions lied in the linear viscoelastic region. In fact, before performing frequency spectra, the linear viscoelastic region was determined and the appropriate strain was selected, by means of strain sweeps conducted at different frequencies (0.1, 1 and 10 Hz) and variable strain ranging from 0.01 to 10 %. This type of test determines the maximum deformation attainable for a system without structural failure. Measurements were performed in triplicate.

3.2.8 Determination of antioxidant activity

3.2.8.1 Effect of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The free-radical scavenging capacity of the sulfated polysaccharides (*Gb*) was analyzed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test according to the method of (Blois, 1958) with some modifications. BHT was used as reference material. Briefly, 0.2 mL of MeOH and 0.3 mL of various sample concentrations (0.1 – 2.0 mg mL^{-1}) dissolved in MeOH were mixed in a 10-mL test tube. DPPH (2.5 mL of 75 μM in MeOH) was then added to achieve a final volume of 3.0 mL. The solution was kept at room temperature for 30 min, and the absorbance at 517 nm (A_{517}) was measured.

The DPPH scavenging effect was calculated as follows:

$$\text{Scavenging effect (\%)} = [A_0 - (A - A_b)/A_0] \times 100\% \qquad \text{Eq. 3.1}$$

Where $A_0 = A_{517}$ of DPPH without sample; $A = A_{517}$ of sample and DPPH; and $A_b = A_{517}$ of sample without DPPH.

3.2.8.2 Hydroxyl radical scavenging activity

The scavenging activity of seaweed polysaccharides against the hydroxyl radical was investigated using Fenton's reaction $(Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH)$. The results were expressed as an inhibition rate. Hydroxyl radicals exhibit a small diffusion capacity and are most reactive in the induction of injuries to cellular molecules and, accordingly, deserve special attention. Hydroxyl radicals were generated using a modified (Smirnoff and Cumbes, 1989) method: in 3 mL sodium phosphate buffer (150 mM, pH 7.4), which contained 10 mM $FeSO_4.7H_2O$, 10 mM EDTA, 2 mM sodium salicylate, 30% H_2O_2 (200 μL) and varying concentrations of polysaccharides (0.1–2.0 mg mL^{-1}). In the control sample, sodium phosphate buffer replaced H_2O_2. The solutions were incubated at 37 °C for 1 h, and the presence of hydroxyl radical was detected by monitoring absorbance at 510 nm.

3.2.9 Statistical analyses

Statistical analyses were performed using Analysis of Variance (ANOVA) and linear regression analysis. The Tukey test (α = 0.05) was used to determine any significance of differences between specific means (SigmaStat, trial version, 2003, USA).

3.3. Results and discussion

3.3.1. Extraction and chemical analyses

The milled red seaweed was washed with water at room temperature and the residue left was exhaustively extracted with hot-water (90 °C). This method revealed to have a higher yield (27.2 %) than other extractions methods (cold extraction) (Table 3.1). Maciel et al. (2008) studied the structural characterization of cold extracted fraction of sulfated polysaccharide from *G. birdiae* and showed that the low yield might be due to the low extraction temperature. However the agar yield of *Gracilaria cornea* from Mexico and from Brazil was found to range from 35.6 to 42.1 % (Freile-Pelegrin and Robledo, 1997) and from 29 to 41 % (Marinho-Soriano, 2001) respectively, depending on the season. Melo et al. (2002) showed that the temperature is one important factor responsible for the yield of extraction.

Table 3.1 – Yield of extraction of agarans obtained from different species of *Gracilaria*

Species	Local	Type of extraction	Yield of extraction (%) ·	Reference
G. birdiae	Brazil	hot extraction	27.2	Present work
G. birdiae	Brazil	room temperature	6.5	(Maciel et al., 2008)
G. bursa-pastoris	France	autoclaving	34.8	(Marinho-Soriano, 2001)
G. cervicornis	Brazil	hot extraction	11.0-20.0	(Marinho-Soriano, 2001)
G. cervicornis	Mexico	hot extraction	25.0-39.3	(Freile-Pelegrin and Murano, 2005)
G. cornea	Brazil	enzymatic digestion	11.0-21.4	(Melo et al., 2002)
G. dura	France	autoclaving	33.5	(Marinho-Soriano, 2001)
G. gracilis	France	autoclaving	11.1-18.7	(Mollet et al., 1998)

· percentages based on milled seaweed

Armisen, (1995) showed that in general the yield of extraction of agar from *Gracilaria* species is very variable due to several factors, such as environmental conditions, seasonal variation, physiological factors and extraction methods. As can be seen in Table 3.2, the extraction with hot-water originated levels of sulfate of 8.4%, higher than those found in the literature for other tropical species of *Gracilaria* (Cote and Hanisak, 1986; Freile-Pelegrin and Murano, 2005; Marinho-Soriano and Bourret, 2005), such as *Gracilaria cornea* (collected in Mexico), which showed variations from 4.8 to 5.5 %. The *G. birdiae* polysaccharide obtained by cold extraction has a sulfate content (6.4 %) (Maciel et al., 2008), in the range observed for polysaccharides from other *Gracilaria* species (2.3-8.9 %). However, Mazumder et al. (2002) found levels of sulfate ranging from 2.1 to 11.7 % for fractions of *G. corticata* and the higher levels were attributed to fractions obtained from cold extraction. It may thus be concluded that low temperatures are responsible for lower agar extraction yields but they allow higher sulfate concentrations to be attained.

Table 3.2 - Chemical analyses and monosaccharide composition of the sulfated polysaccharide obtained from *G. birdiae*

G.birdiae Temperature of extraction (°C)	Weight (g)	Carbohydrate (%)	Sulfate [a] (%)	Protein (%)	Monosaccharide composition [b] (mol %)			
					AnGal	6-Gal	3- and 4-Gal	Gal
90	20.0	85.6	8.4	2.5	25.06	9.2	(*tr*)	65.4

[a] Expressed as Na_2SO_3.

[b] AnGal correspondes to 3,6-anhydrogalactose ; 3-Gal to 3-*O*-methylgalactose, 4-Gal to 4-*O*-methylgalactose, 6-Gal to 6-*O*-methylgalactose and *tr* = traces (< 1 mol %)

In the present work *Gb* exhibits low levels (2.5 %) of protein content (Table 3.2), which are in agreement with the values observed in the literature (Melo et al., 2002). Analyses of total sugars by the method of phenol/sulfuric acid according to Dubois et al. (1956) showed high percentages of these compounds (85.6 %).

3.3.2. Monosaccharide composition and molar mass distribution

Table 3.2 shows the monosaccharide composition of galactans obtained from *G. birdiae*. The results show that these galactans have a high content of galactose (65.4 %) and 3,6-anhydrogalactose (25.1 %) obtained by reductive hydrolysis and quantified by GC. The methyl derivatives: 6-*O*-methyl-galactose (9.2 %) and in smaller quantities 3-*O*- and 4-*O*-methyl-galactose (0.33 %) were quantified by GC-MS. The presence of galactose and 3,6-anhydrogalactose as major monosaccharides has been observed in several other algae of the order *Gracilariales* (Melo et al., 2002; Mollet et al., 1998) and their quantities are generally variable.

In order to estimate the peak molar mass (M_{pk}) for *Gb* polysaccharide, a calibration was obtained using pullulan fractions. The equation obtained from the calibration plot was:

Log M_w = 13.142 – 0.964 V_e Eq. 3.2

Where V_e is the elution volume in mL. The linear correlation coefficient was 0.9991.

The GPC chromatogram is shown in Figure 3.1. A peak at 7.86 mL and a shoulder at 6.98 mL were detected. *Gb* polysaccharide behaves as a heterogeneous system similar to other seaweeds' polysaccharides, such as those from *G. cornea* (Melo et al., 2002) and *Botryocladia occidentalis* (Farias et al., 2000).

Based on Eq. 3.2, *Gb* polysaccharide shown M_{pk} values of 2.6 x10^6 and 3.7 x 10^5 g/mol, corresponding to the identified shoulder and peak. This high molar mass can be justified by grouping of polysaccharide chains, which can also be observed by the highly heterogeneous profile.

Both synthetic polymers and naturally occurring polysaccharides are polydisperse, meaning that in general they do not have sharply defined molecular weights, but rather average molecular weights representing a distribution of molecular species nearly identical in structure but of varying chain length (Stanley, 2006) .

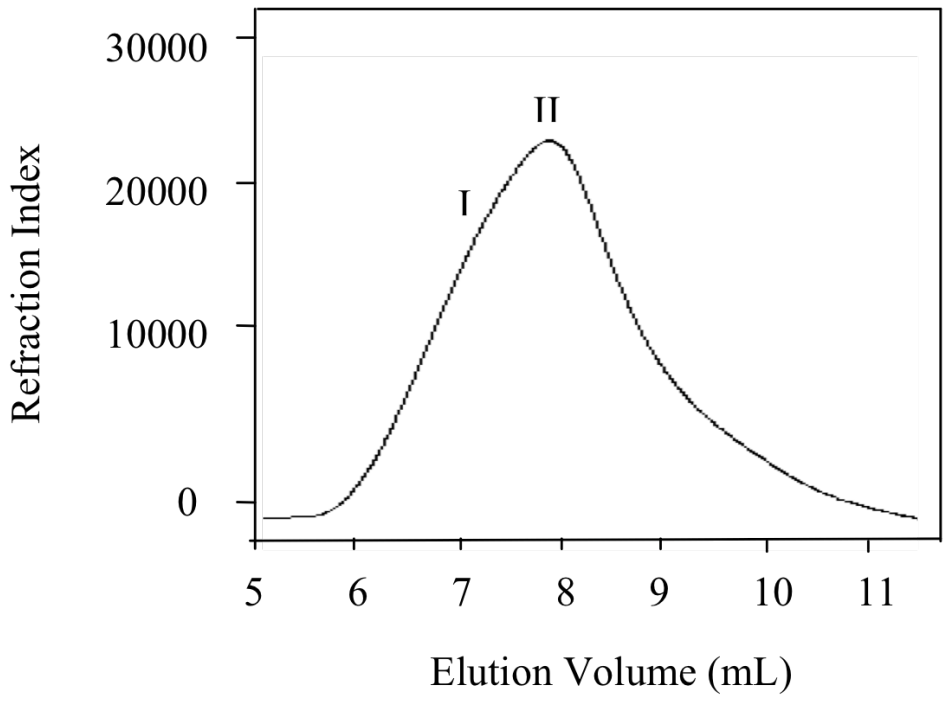

Figure 3.1 - GPC curve for *G. birdiae* sulfated polysaccharide. I = Shoulder; II = Peak.

3.3.3. FTIR analysis

Table 3 shows results from FTIR analyses performed on *Gb* polysaccharide and reveals the presence of most characteristic bands of polysaccharides from red seaweeds.

The regions of the spectrum between 1400 and 700 cm^{-1} were expanded to better identify the positions of sulfate groups present in these polysaccharides (Figure 3.2). The most important bands were those found at 1375 cm^{-1} and 1255 cm^{-1}, corresponding to ester sulfate groups; the region around 1072 cm^{-1} and 890 cm^{-1} are equivalent to the skeleton of galactans and agar specific band, respectively. The region around 931 cm^{-1} can be attributed to the C-O-C group of 3,6-anhydro-α-L-galactopyranose and the absorption around 855 cm^{-1} indicates the presence of sulfate groups on the C-4 of galactose. The band around 820 cm^{-1} is attributed to the 6-sulfate group of D-galactose units.

Studies with algae *G. corticata*, *G. domingensis*, *G. mammillaris* and *G. pseudoverrucosa* showed the presence of the band around 820 cm^{-1} and the absence of the band around 855-840 cm^{-1}. However, after alkaline treatment, the infrared spectrum of the same polysaccharide registered the presence of a band around 850-840 cm^{-1} and no band around 820 cm^{-1}. It was also observed that the same alkaline treatment resulted in an increase of the bands intensity around 930 cm^{-1}. This means that the band around 820 cm^{-1} is related to the presence of the sulfate group at C-6 of L-galactose units, which are converted to 3,6-anhydrogalactose after alkaline treatment (Mazumder et al., 2002). The residues of 6-sulfate-α-L-galactose are known to be precursors of 3,6-anhydro-α-L-galactose (Talarico et al., 2004).

Melo et al. (2002) also found bands at 1370 and 770 cm^{-1} and Maciel et al. (2008), when studying the cold extraction of *G. birdiae* polysaccharide, observed the same bands characteristic of agarocolloids (1375 and 775 cm^{-1}).

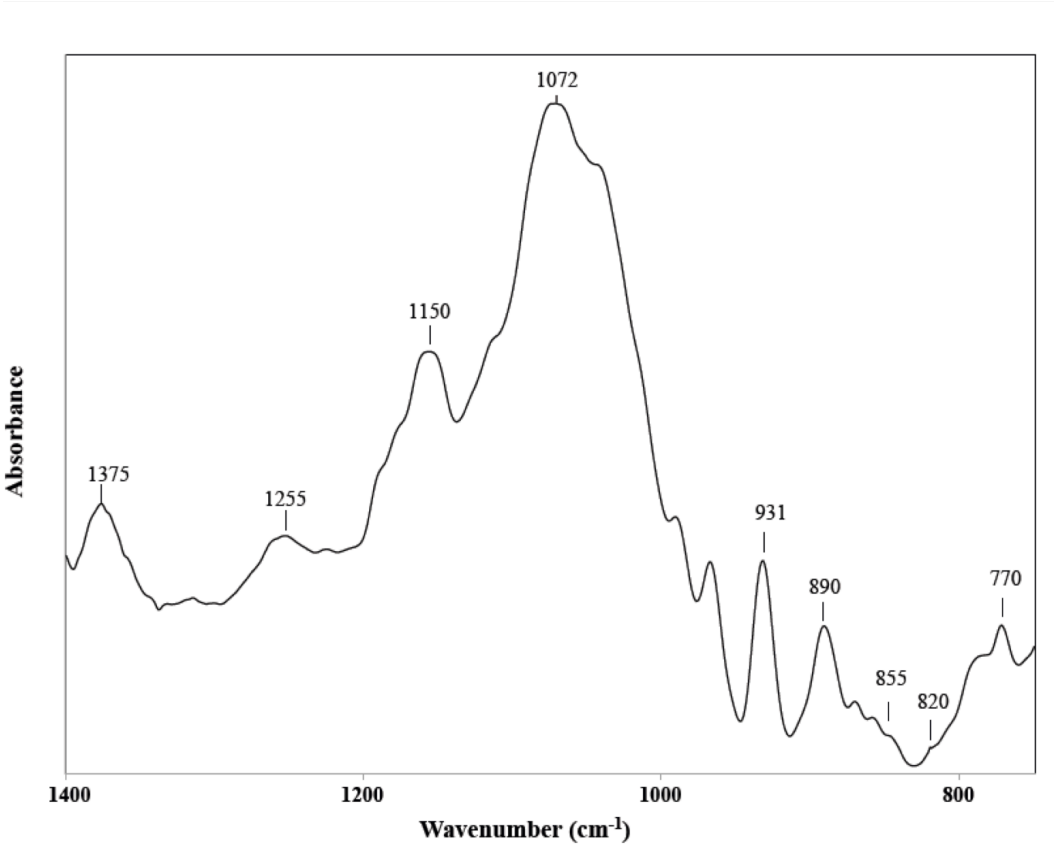

Figure 3.2 - FT-IR Spectra of sulfated polysaccharide of *G. birdiae.*

3.3.4. Rheological characterization

The rheological behaviour of *G. birdiae* sulfated polysaccharide was compared to that of commercial agar, a gel-forming polysaccharide thoroughly used by the food industry. The mechanical spectra obtained for *Gb* sulfated polysaccharide and for commercial agar at 1.5 % (w/w) and at 25 °C are presented in Figures 3.3 a) and b), respectively.

Both polysaccharides exhibit a gel-like behavior: storage modulus (*G'*) higher than loss modulus (*G''*) in the whole range of frequencies covered and with both moduli being almost frequency independent. The mechanical spectra of the two sulfated polysaccharides are similar however, for the same concentration, the commercial agar solution shows a higher elastic behavior that can be due to the higher purity of the commercial sample (> 95%). Also, as expected for gels, the plot of $[\eta^*]$ *versus* log ω was linear with a slope of -1 (Figure 3.3).

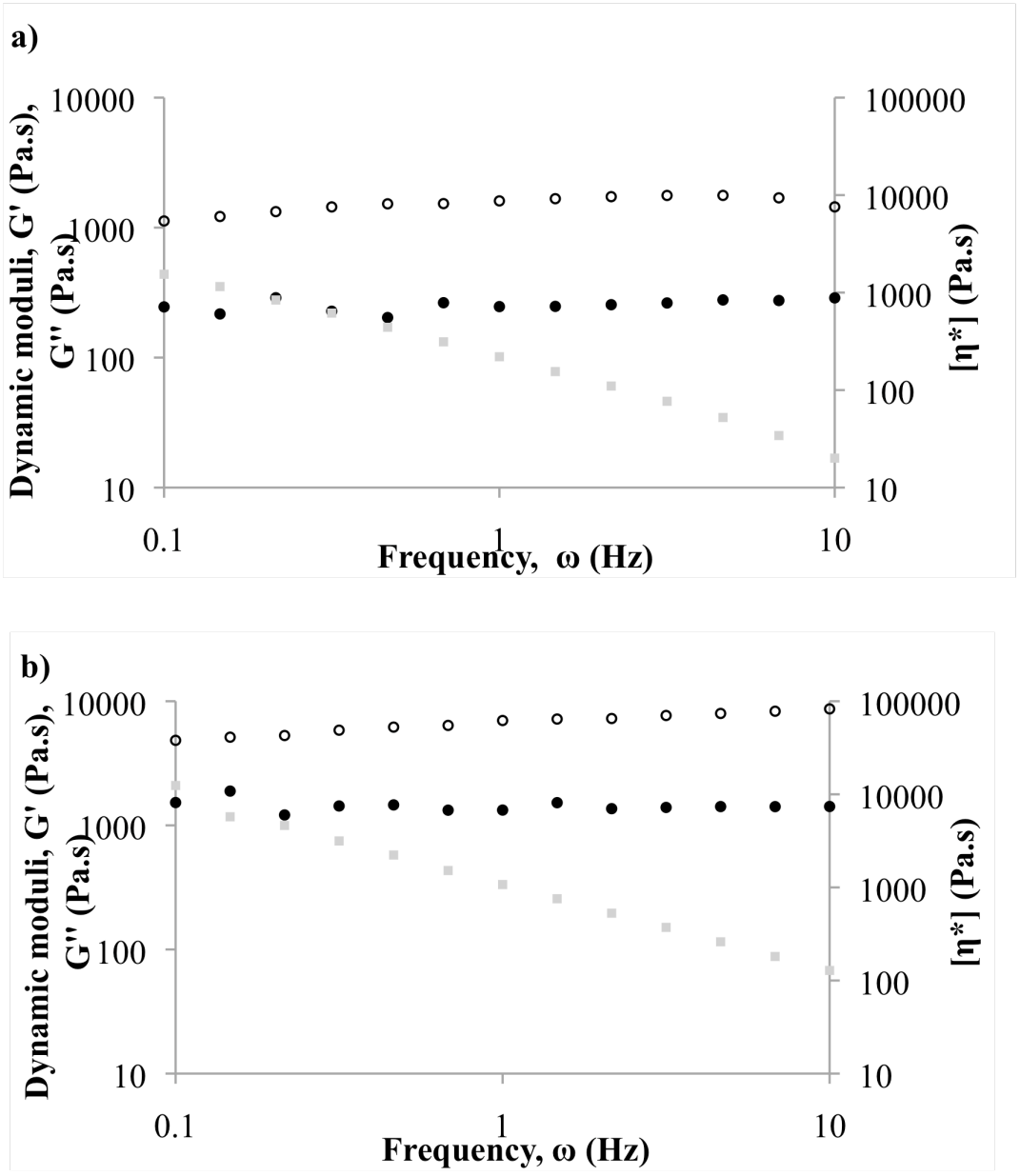

Figure 3.3 - Mechanical spectra of a) *G. birdiae* sulfated polysaccharide and b) commercial agar at a total polysaccharide concentration of 1.5 %, at 25 °C: (G' (○), G'' (●) and [η^*] (■)).

Several authors reported that the 3,6 anydro-α-L-galactopyranose (AnGal) content in *Gracilaria* agars is an important component in the control of gel textural quality (Lahaye and Rochas, 1991). Also, Bird et al. (1981) observed that high gel strength of agar sample is associated with high molecular weight and larger polymers and these characteristics reflect a high capability to form 3-dimensional lattices

between water and gel helices. The polysaccharide extracted in this work exhibits a solid-like behavior, that can be due to the high content in 3,6 AnGal (25.06 mol %). Rodríguez et al. (2009) characterized the polysaccharides isolated from *Gracilaria gracilis* at three temperatures and observed that the polysaccharide extracted at 90 °C exhibited an 3,6 AnGal content of 35 - 36 mol % and formed a gel with high strength.

3.3.5. Free-radical scavenging activity

DPPH is a free-radical compound that has been widely used to determine the free-radical scavenging ability of samples (Qi et al., 2005; Wang et al., 2009). This method allows determining the anti-radical activity of an antioxidant by measuring the decrease in absorbance of the DPPH radical caused by the scavenging of the hydroxyl radical through hydrogen donation. In this work, DPPH free-radical scavenging effect of each sample was measured (Figure 3.4a). Results demonstrated that the sulfated polysaccharide had a noticeable effect on inhibiting the formation of these radicals (IC_{50} = 1.62 mg mL^{-1}). However, none of the samples had stronger activity than BHT at the same concentration.

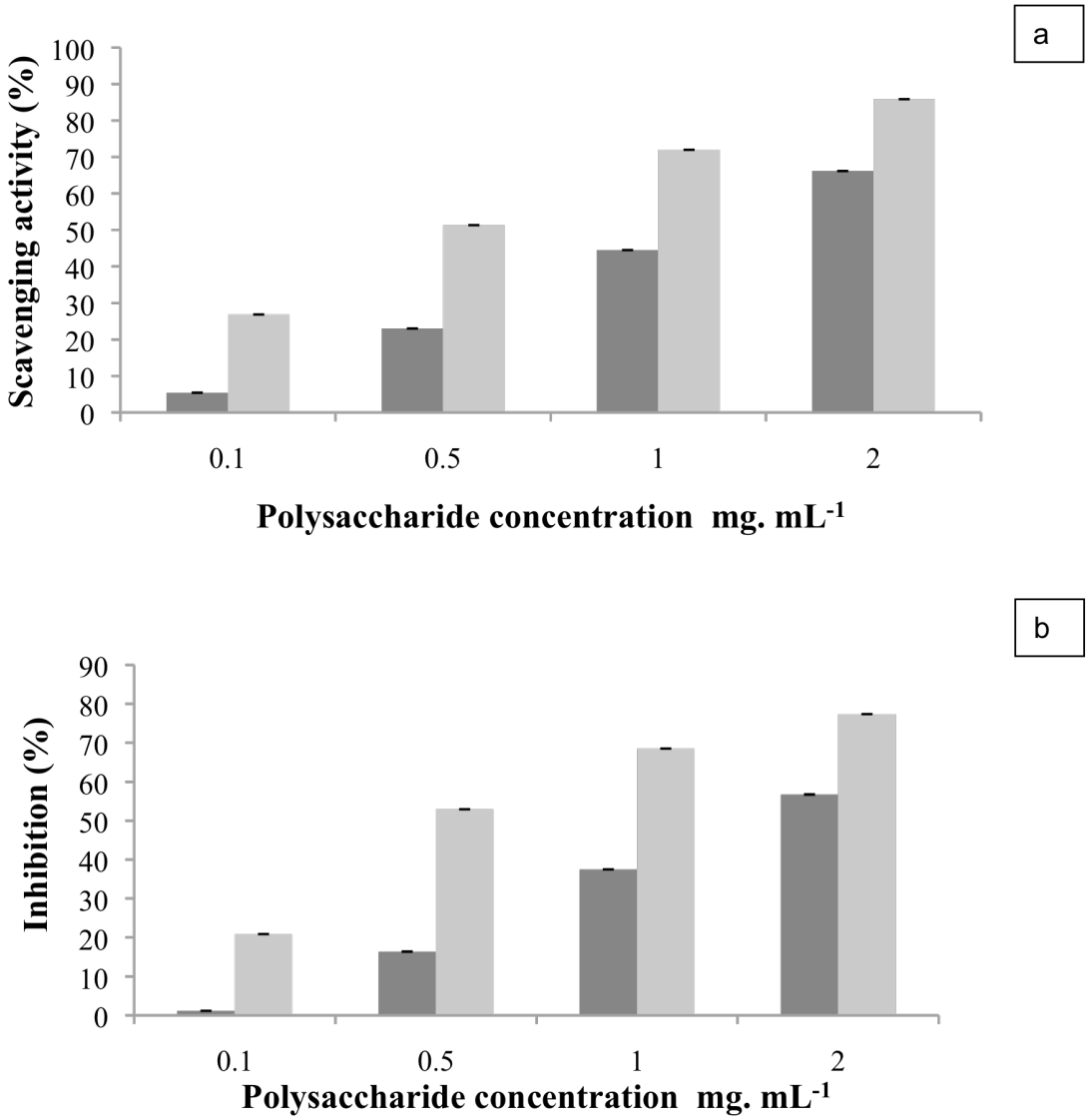

Figure 3.4 - Antioxidant activity: a) Effect of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and b) Inhibition of hydroxyl radicals effects by sulfated polysaccharide of *G. birdiae*. ■ Gb ; ■ BHT as a positive control. Values are means ± SD (*n*=3).

The results obtained for the inhibition of hydroxyl (OH·) radical formation demonstrated that scavenging activity of *Gb* polysaccharide increased significantly ($p < 0.05$) for higher polysaccharide concentrations. The IC_{50} value of *Gb* eliminating OH· was about 1.73 mg mL^{-1}, which indicates that the scavenging activity of *Gb* against OH· was less than that of BHT). Qi et al. (2005) concluded that the OH· scavenging activity of different polysaccharides was related to the presence of the same structural feature in which

all of the polysaccharides had one or more –OH and -OSO_3H groups in the molecule. These results proved that sulfate content had a significant effect on OH^- scavenging activity. Souza et al. (2007) showed that iota-carrageenan had a higher inhibitory effect on OH^- formation in relation to the lambda- and kappa-carrageenans. Several works have demonstrated that the presence of sulfate groups in seaweed polysaccharides is responsible for numerous types of biological activities, such as antioxidant activities (Qi et al., 2005; Wang et al., 2009).

3.4. Conclusion

The sulfated polysaccharide from marine red algae *Gracilaria birdiae* is composed of galactose (65.4 %) and methyl derivatives 6-*O*-methyl-galactose (9.2 %) and in smaller quantities *3-O-* and *4-O*-methyl-galactose (0.33 %). This polysaccharide also presents a high content of 3,6-anhydrogalactose (25.6 %) and has a sulfate content of 8.4 %.

The sulfated polysaccharide of *Gb* characterized by FTIR exhibits the characteristic bands of agarocolloids (at 1375 and 770 cm^{-1}).

It has also been shown that the *G. birdiae* sulfated polysaccharide is a promising agent to be evaluated for the application in the food industry, and that it presents a significant antioxidant activity.

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Chapter 4 - Polysaccharide of seaweeds as edible coatings for foods

4.1 Introduction

The food and packaging industries have been joining efforts to reduce the amount of food packaging materials, once the environmental issues became important to the consumer. As an answer to that concern, several problems were addressed in order to foster the commercial use of bio-based primary food packaging materials. These problems include degradation rates under various conditions, changes in mechanical properties during storage, potential for microbial growth, and release of harmful compounds into the packaged food product (Lin and Zhao, 2007). On the other hand, consumers around the world demand for food of high-quality, without chemical preservatives, and with an extended shelf life. Therefore, an increased effort has been made to discover new natural preservatives and antimicrobials (Lin and Zhao, 2007).

The future generation of packaging materials will be derived from renewable resources. These materials will ideally be biodegradable. However, natural polymeric materials vary in their rate of degradation in the environment, and some proteins, for example, cannot presently be classified as degradable because of standard definitions (Lin and Zhao, 2007). Edible films and coatings can improve shelf-life and food quality by providing good and selective barriers to moisture transfer, oxygen uptake, lipid oxidation, losses of volatile aromas and flavors (Kester and Fennema, 1986), better visual aspect, and reduction of microbiological contamination.

Agar, which exists in algae as a gel at the temperature of the natural environment, is a gelatinous product from the red algae class (*Rhodophyceae*). Agar is a heterogeneous complex mixture of related polysaccharides having the same backbone chain structure. The main components of the chain are D-galactopyranose and 3,6-anhydro-L-galactopyranose, which alternate through α-(1,4) and β-(1,3) linkages. Agar is lightly sulfated; the main fractions are agarose, a neutral polymer, and agaro-pectin, a sulfated polymer. In addition, the charged chains have pyruvic acid bound in ketal form. Depending on the source of the agar, the molecular weight of the chains varies from 80 000 to 140 000 Da. Agar is insoluble in cold water and slightly soluble in ethanolamine, whereas in the dried state, it is soluble in hot water (Araki, 1966; Marinho-Soriano and Bourret, 2003).

Polysaccharide film-forming materials such as starch and starch derivatives, cellulose derivatives, alginate, carrageenan, chitosan, pectinate, and various gums have been studied extensively for the development of edible packaging. Indeed, agar is usually used as a gel builder in candy and desserts.

The low cost and the difference in their gel-forming behaviors are the main driving forces inducing investigations on new applications of these polysaccharides (Cardozo et al., 2007).

Cheese is a complex food product consisting mainly of casein, fat and water. Several researchers have recommended that fresh cheeses (e.g. cream cheese, decorated cream cheese, soft cheese, and cottage cheese) be packaged in modified atmospheres with N_2 and/or CO_2 replacing the O_2 in the package (Mannheim and Soffer, 1996). However spoilage caused by yeast and especially bacteria may still occur even at very low O_2 and elevated CO_2 levels (Westall and Filtenborg, 1998). Semi-soft and hard cheeses (whole, sliced or shredded) have a relatively high respiration rate, which require a packaging material somewhat permeable to CO_2 to avoid blowing of the packaging. Meanwhile, O_2 must be kept out to avoid fungal spoilage and oxidation of the cheese. Instead, these products require a balanced oxygen and carbon dioxide atmosphere to prolong their shelf-life (Haasum and Nielsen, 1998).

In semi-hard cheeses the factor that most affects cheese stability is the water activity (a_w), which depends mainly on moisture and salt contents. During ripening, a_w is not constant but decreases until the cheese surface is in equilibrium with the surrounding atmosphere, thus influencing the microbiological and chemical evolution of the cheese (Saurel et al., 2004). Additional environmental factors must be considered in selecting a material for cheese coating (e.g. the light). All these factors affect not only cheese's physical characteristics but also its flavor during storage. In fact, many different compounds contribute to cheese flavor and most of them form during cheese ripening (Robertson, 2006).

These are the reasons why cheese was chosen as a model food in this study. The cheese studied in this work is a cylindrical, yellow and semi-hard cheese; it is sold unpackaged, covered with a synthetic/antibiotic coating, and under normal storage conditions it suffers an excessive water loss. The present work evaluates the possibility of using functional polysaccharides as coatings on semi-hard cheese. The choice of the best coating is made taking into consideration its wettability, permeability's and opacity properties.

4.2. Materials and methods

4.2.1 Materials

Edible coating solutions were prepared with: agar extracted from *Glacilaria birdiae* seaweed (specimens of the red seaweed *G. birdiae* were collected in 2006 on the Atlantic coast of Brazil, Fleixeiras, Trairi – Ceará); corn oil (Sovena, Portugal); glycerol 87 % (Panreac, Spain) and sorbitol 97 % (Acros Organics, Belgium); Tween 80 (Acros Organics, Belgium); lactic acid (Merck, Germany) and distilled water. A commercial semi-hard cheese was obtained from Queijo Saloio S.A. (Portugal) without any previous treatment (without ripening and coating), two days after production, the samples being stored at 5 °C and 80 % RH until further use. *Regional Saloio* cheese is a full fat cheese produced with a mixture of caprine, bovine and ovine pasteurized milk which, after coating with a synthetic coating and an antibiotic protector, is submitted to a short ripening period at low temperatures (5 °C and 12 °C in different stages of the ripening process). It requires conditions of 0-22 °C for sale. The cheese physicochemical composition is: moisture 46 %, fat 25 %, protein 18.4 %, total ash 3.58 %, chlorides 1.54, pH 4.8 and total acidity 1.40 (Pantaleão, 2007).

4.2.2 Polysaccharide extraction

Specimens of the red seaweed *G. birdiae* were collected in the Atlantic coast of Brazil (Fleixeiras Beach, Trairi, Ceará). This species grows attached to rocks or dead coral. The diploid phase that develops directly on the female thallus, the carposporophyte, is evident all year in the area, and was selected as seed material. The seedlings were cleaned and then tied in a structure made of string, which was placed in the sea (03° 13´ 25" S and 039° 16´ 65" W), where it was anchored and submerged for two months. After that period algae were collected, cleaned of epiphytes, washed with distilled water and stored at - 20 °C.

The samples were air dried and then milled. The powder was extracted with water (1.5 % w/v) at 25 °C with mechanical stirring for 15 h. The algal residue was removed by filtration and supernatant discarded. The algal residue obtained from the first extraction was extracted with water at 90 °C with mechanical stirring for 45 min. The residue was removed by centrifugation, and the supernatant was precipitated with ethanol (1:3 v/v), lyophilized and stored.

4.2.3 Coating and film preparation

The coating formulations were based in a two level factorial design with polysaccharide concentrations of 0.5 % and 1.5 % (w/v), plasticizer concentrations of 0.5 % and 2.0 % (v/v) and oil concentration of 0 % and 0.5 % (w/v).

The coating solutions from agar of *G. birdiae* (*Gb*) were prepared dissolving the agar (0.5 or 1.5 % w/v) in distilled water with agitation using a magnetic stirrer during 20 minutes at 60 °C. As plasticizers, glycerol and a mixture of glycerol/sorbitol (50:50) were added in concentrations between 0.5 and 2.0 % (w/v). Corn oil was added in a concentration of 0.5 % (w/v). In all the cases a constant amount (13 mL) of solution was cast onto a 5.7 cm diameter glass plate in order to maintain the film thickness. The films were dried in an oven at 35 °C during 16 hours. These solutions correspond to solutions 1 to 16, in Table 4.1. Films were maintained at 20 °C and 50 % RH before permeability and opacity tests.

4.2.4 Film thickness.

The film thickness was measured with a digital micrometer (No. 293-561, Mitutoyo, Japan). Five thickness measurements were taken on each testing sample in different points and the mean values were used for calculation of water vapor permeability (*WVP*), oxygen permeability (O_2P) and dioxide carbon permeability (CO_2P).

4.2.5 Critical surface tension and surface tension of cheese skin

According to Zisman, (1964), in systems having a surface tension lower than 100 $mN \cdot m^{-1}$ (low-energy surfaces), the contact angle formed by a drop of liquid on a solid surface will be a linear function of the surface tension of the liquid, γ_{Lv}, (where phase *V* is air saturated with the vapor of liquid, *L*). The Zisman method, briefly described below, is applicable only for low energy surfaces; therefore it is necessary to determine the surface energy of the cheese.

For a pure liquid, if polar (γ_L^p) and dispersive (γ_L^d) interactions are known, and if θ is the contact angle between that liquid and a solid, the interaction can be described in terms of the reversible work of adhesion, *Wa*, as:

$$W_a = W_a^d + W_a^p \Leftrightarrow W_a = 2\cdot\left(\sqrt{\gamma_S^d\cdot\gamma_L^d} + \sqrt{\gamma_S^p\cdot\gamma_L^p}\right) \quad \text{Eq.4.1}$$

Where γ_S^p and γ_S^d are the polar and dispersive contributions of the surface of the studied solid. Rearranging equation 4.1, yields:

$$\frac{1+\cos\theta}{2}\cdot\frac{\gamma_L}{\sqrt{\gamma_L^d}} = \sqrt{\gamma_S^p}\cdot\sqrt{\frac{\gamma_L^p}{\gamma_L^d}} + \sqrt{\gamma_S^d} \quad \text{Eq. 4.2}$$

For a pure liquid, if polar and dispersive interactions are known, and if the contact angle between that liquid and a solid is obtained, the interaction can be described by:

$$W_a = W_a^d + W_a^p \Leftrightarrow W_a = 2\cdot\left(\sqrt{\gamma_S^d\cdot\gamma_L^d} + \sqrt{\gamma_S^p\cdot\gamma_L^p}\right) \quad \text{Eq.4.3}$$

$$\frac{1+\cos\theta}{2}\cdot\frac{\gamma_L}{\sqrt{\gamma_L^d}} = \sqrt{\gamma_S^p}\cdot\sqrt{\frac{\gamma_L^p}{\gamma_L^d}} + \sqrt{\gamma_S^d} \quad \text{Eq 4.4}$$

The contact angle determinations of at least three pure compounds: bromonaphthalene (Merck, Germany), formamide (Merck, Germany) and ultra pure water, on the surface of the cheese (cheese skin) combined with the values presented below, will allow the calculation of both the independent variable, $\left(\sqrt{\frac{\gamma_L^p}{\gamma_L^d}}\right)$, and the dependent variable, $\left(\frac{1+\cos\theta}{2}\cdot\frac{\gamma_L}{\sqrt{\gamma_L^d}}\right)$, from equation 4.4.

The surface tension, the dispersive and the polar component were, respectively, 72.10, 19.90 and 52.20 mN·m^{-1} for water, 44.40, 44.40 and 0.00 mN·m^{-1} for bromonaphtalene and 56.90, 23.50 and 33.40 mN·m^{-1} for formamide (Busscher, 1984).

The estimation of the critical surface tension (γ_c) was performed by extrapolation from Zisman plots (Zisman, 1964). Zisman plots have long been used to characterize the wettability of low-energy surfaces. Zisman plots are obtained by plotting the cosine of the contact angle of pure liquids on a solid surface to be studied against the surface tension of the same series of liquids. The intercept of these curves with cos q = 1 is known as the critical surface tension (γ_c). The critical surface tension is an imaginary point of the γ_{sv} value and it is frequently used to describe the wettability of a surface.

It represents the value of γ_{LV} of a liquid above which the spreading of this liquid in a solid surface is complete. The critical surface tension (γ_c) is defined as:

$$\gamma_c = \lim \gamma_{LV} \quad \text{as } \theta \rightarrow 0 \qquad \text{Eq. 4.5}$$

All experiments were performed at 21.3 ± 0.2 °C with 20 replicates for each of the compounds used.

4.2.6 Wettability

The wettability was studied by determining the values of the spreading coefficient *Ws* and the works of adhesion (*Wa*) and cohesion (*Wc*). The adhesive forces promote the liquid spreading on a solid surface and the cohesive forces promote their contraction. The wetting behaviour of the solutions will mainly depend on the balance between these forces. The surface tension of the coating solution was measured by the pendant drop method using the Laplace-Young approximation (Song and Springer, 1996).

The contact angle (θ) of a liquid drop on a solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions: solid-vapor (γ_{SV}), solid-liquid (γ_{SL}), and liquid-vapor (γ_{LV}). The equilibrium spreading coefficient Ws is defined by equation 4.6 (Rulon and Robert, 1993) and can only be negative or zero.

$$Ws = Wa - Wc = \gamma_{SV} - \gamma_{LV} - \gamma_{SL} \qquad \text{Eq. 4.6}$$

Where *Wa* and *Wc* are the works of adhesion and cohesion, defined by equation 4.7 and equation 4.8, respectively.

$$Wa = \gamma_{LV} + \gamma_{SV} - \gamma_{SL} \qquad \text{Eq. 4.7}$$

$$Wc = 2 \cdot \gamma_{LV} \qquad \text{Eq. 4.8}$$

Contact angle (θ) and liquid-vapour surface tension (γ_{LV}) were measured in a face contact angle meter (OCA 20, Dataphysics, Germany). The samples of the coatings were taken with a 500 mL syringe (Hamilton, Switzerland), with a needle of 0.75 mm of diameter. The contact angle at the cheese surface was measured by the sessile drop method (Newman and Kwok, 1999).

Measurements were made in less than 30 s. Ten replicates of contact angle and surface tension measurements were obtained at 21.3 ± 0.5 °C.

4.2.7 Water vapor permeability measurement (*WVP*)

The measurement of water vapor permeability (*WVP*) was determined gravimetrically based on ASTM-D-3985-02 (2002) method. The film was sealed on the top of a permeation cell containing distilled water (100 % RH; 2337 Pa vapor pressure at 20 °C), placed in a desiccator at 20 °C and 0 % RH (0 Pa water vapor pressure) containing silica. The cells were weighed at intervals of 2 hours during 10 hours. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside the test cell by using a miniature fan inside the desiccator. The slope of weight loss versus time was obtained by linear regression. Three replicates were obtained for each film.

4.2.8 Oxygen and carbon dioxide permeability

Oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) were determined based on the ASTM-D-3985-02 (2002) method. The films were sealed between two chambers, having each one two channels. In the lower chamber O_2 (or CO_2) was supplied at a controlled (J & W Scientific, ADM 2000, USA) flow rate to keep its pressure constant in that compartment. The other chamber was purged by a stream of nitrogen, also at controlled flow. Nitrogen acted as a carrier for the O_2 (or the CO_2).

In the case of O_2P measurement, the flow leaving this chamber was connected to an O_2 sensor (Mettler Toledo, Switzerland), which measured the O_2 concentration in that flow on-line. In the case of CO_2P measurement the flow leaving this chamber was collected in a syringe for CO_2 quantification. To determine CO_2 concentration, 1 mL of sample was injected in a gas chromatograph (Chrompack 9001, Middelburg, Netherlands) at 110 °C with a column Porapak Q 80/ 100 mesh 2 m x 1/8" x 2 mm SS, using a flame ionization detector (FID) at 110 °C. Helium at 23 $mL \cdot min^{-1}$ was used as carrier gas. A standard mixture containing 10 % CO_2, 20 % O_2 and 70 % N_2 was used for calibration.

The flows of the two chambers were connected to a manometer to ensure the equality of pressures (both at 1 atm) between both compartments. As the O_2 (and the CO_2) was carried continuously by the nitrogen flow, it was considered that O_2 (and the CO_2) partial pressure in the upper compartment is null, therefore ΔP is equal to 1 atm. Three replicates were obtained for each sample, in each case (O_2P and CO_2P).

4.2.9 Opacity

The opacity of the samples was determined according to the Hunter lab method, with a Minolta colorimeter (CR 400; Minolta, Japan), as the relationship between the opacity of each sample on the black standard (Y_b) and the opacity of each sample on the white standard (Y_w).

4.2.10 Statistical analyses

Statistical analyses were performed using Analysis of Variance (ANOVA) and linear regression analysis. The Tukey test (α = 0.05) was used to determine any significance of differences between specific means (SigmaStat, trial version, 2003, USA).

4.3 Results and discussion

4.3.1 Critical Surface Tension and Surface Tension of cheese

The determination of the surface tension and of the critical surface tension of the cheese allows the characterization of the surface of its skin. According to Zisman, (1964), in systems having a surface tension lower than 100 $mN \cdot m^{-1}$ (low energy surfaces), the contact angle formed by a drop of liquid on a solid surface will be a linear function of the surface tension of the liquid, γ_{LV}, (where phase *V* is air saturated with the vapor of liquid, *L*), which allows the application of the method to determine the wettability.

The surface from the cheese displays values of critical surface and surface tension of 18.33 ± 0.10 $mN \cdot m^{-1}$ and 37.79 ± 0.76 $mN \cdot m^{-1}$ respectively. The cheese surface is a low-energy surface (< 100 $mN \cdot m^{-1}$) and presents a higher dispersive component (29.93 ± 0.41 $mN \cdot m^{-1}$), which shows its ability to participate in non-polar interactions, and a low polar component (7.87 ± 0.37 $mN \cdot m^{-1}$). A surface with these characteristics interacts with liquid primarily by dispersion forces, influencing the effective spreading of the coating on the cheese surface. The compatibility of the polarity (apolar or polar) of the surface and of the coating may play therefore an important role in the wettability of the surface. The cheese, being very rich in apolar components (e.g. fat) features a significant apolar influence.

4.3.2 Wettability

The wettability was studied by determining the values of the spreading coefficient Ws. Wettability is one of the most important properties when evaluating the capacity of a solution to coat a designated surface. In practical terms, the closer the *Ws* values are to zero, the better a surface will be coated. The results show (Table 4.1) that depending of the amount of polysaccharide, plasticizer and oil added, the values of *Ws* are statistically different. Considering the solutions tested, the best (higher) value of *Ws* on cheese surface was determined (Tukey test, $p < 0.05$). Solution 11 was the best, presenting statistically significant differences from the other samples (Table 4.1). As in previous cases, the solutions containing oil present the best value of *Ws.* When there were no statistically significant differences between polysaccharide solutions, it has been assumed that both were equally good in terms of wettability and that their differentiation must be made based on other criteria (such as water vapor, O_2 and CO_2 permeability and opacity).

Table 4.1 - Spreading coefficient *Ws* obtained for the tested polysaccharide solutions on cheese.

Solution	Polysacch. Solutions (w/v)	Glycerol (w/v)	Glycerol/ Sorbitol (w/v)	Oil (w/v)	*Ws* (mN/m)
1	0.5	0.5	-	-	-45.85±3.27^{a}
2	0.5	2.0	-	-	-36.49±2.65^{bc}
3	0.5	0.5	-	0.5	-55.46±2.33^{d}
4	0.5	2.0	-	0.5	-47.37±1.81^{ae}
5	0.5	-	0.5	-	-49.62±1.62^{e}
6	0.5	-	2.0	-	-45.69±2.46^{f}
7	0.5	-	0.5	0.5	-52.81±2.34^{d}
8	0.5	-	2.0	0.5	-47.97±1.81^{e}
9	1.5	0.5	-	-	-39.24±1.83^{gh}
10	1.5	2.0	-	-	37.61±2.16^{cgh}
11	1.5	0.5	-	0.5	-30.45±1.39^{j}
12	1.5	2.0	-	0.5	-37.52±1.38^{cg}
13	1.5	-	0.5	-	-43.97±2.85^{fi}
14	1.5	-	2.0	-	-46.87±1.50^{a}
15	1.5	-	0.5	0.5	-34.50±3.41^{bj}
16	1.5	-	2.0	0.5	-40.88±1.14^{hi}

Values reported are the means ± standard deviations (n = 20, 95 % confidence interval, at 21.4 ± 0.5 °C). Different superscript letters in the same column indicate a statistically significant difference (Tukey test $p < 0.05$).

4.3.3 Water vapor permeability (*WVP*)

The water vapor permeability is the most extensively studied property of edible films mainly because of the importance of the water in deteriorative reactions. The three best solutions in terms of wettability were subsequently analyzed for *WVP*.

Table 4.2 also shows the values of *WVP* for the best solutions of *G. birdiae* (Gb2, Gb11, and Gb15). The lower *WVP* values were registered for films from solutions Gb11 and Gb15, which are not statistically different, but have a statistically significant difference with solution Gb2. An increase of the concentration of polysaccharides corresponds to a decrease of *WVP*, presumably due to a stronger gel network, where the polysaccharide molecules are closer together. Furthermore, the solution with sorbitol (Gb15) showed the lowest value of *WVP*; this observation may be explained by the larger size and lower hygroscopicity of the sorbitol compared to glycerol, reducing its ability to affect hydrogen bonding between polysaccharide chains (Hong, 2003). Kester and Fennema, (1986) showed that the plasticizer decreases the intermolecular attractions between polymeric chains, facilitating the penetration of polar water vapor molecules. Glycerol is a hydrophilic molecule (polar) and an increase of its concentration causes an increase of water vapour mass transfer. Being apolar, CO_2 and O_2 possibly do not penetrate so easily in such a polar moiety.

The addition of oil promoted a decrease of *WVP* in *G. birdiae* films. In this line, Hernandez-Munõz et al. (2004) indicated that *WVP* occurs through the hydrophilic portion of the film, therefore depending of the hydrophilic-hydrophobic ratio of the films. Avena-Bustillos and Krochta, (1993) showed that *WVP* decreases with the addition of beeswax to sodium caseinate films. Also (Péroval et al., 2002), showed that arabinoxylan films with hydrogenated palm oil have lower *WVP* values than films without oil. Pranoto et al. (2005) showed similar results with alginate-based films containing garlic oil.

4.3.4 Oxygen permeability (O_2P), Carbon dioxide permeability (CO_2P) and Opacity

Oxygen is a key factor in cheese preservation. Films, which provide a proper oxygen barrier, can help improving food quality and extending food shelf life. Table 4.2 presents the values of O_2P of the analyzed samples. There were no statistically significant differences for the films from solutions of *G. birdiae* in terms of O_2P (Table 4.2), however, the samples with higher concentration of plasticizer have higher values of O_2P than the samples with lower concentration, which were also shown by (Caner et al., 1998).

The plasticizer decreases the intermolecular attractions between polymeric chains, facilitating the penetration of gas molecules (Kester and Fennema, 1986). However, the partial replacement of glycerol by sorbitol provoked a decrease of the O_2P value, this difference can be explained by the different molecular size and hygroscopicity of sorbitol and glycerol (Hong, 2003).

G. birdiae films display a very significant decrease of the value of CO_2P with the increase of polysaccharide concentration. Also here, the addition of sorbitol decreases the value of CO_2P, as shown by Garcia et al. (2000). The effect of polysaccharide concentration seems to be, by far, the most important one affecting CO_2P. It is known that the addition of plasticizer decreases the presence of cracks and pores, improving the dispersion and decreasing the gas permeability (Garcia et al., 2000), thus the results shown here can be explained in light of these facts. Similar results were obtained for carbon dioxide permeability (CO_2P).

Table 4.2 - Values of water, O_2, CO_2 permeability and opacity of the films.

Solution		$WVP \times 10^{-11}$ (g·(m·s·Pa)$^{-1}$)	$O_2P^{*} \times 10^{-15}$ (g·m·(Pa·s·m^2)$^{-1}$)	$CO_2P \times 10^{-15}$ (g·m·(Pa·s·m^2)$^{-1}$)	Opacity* (%)
G. birdiae	Gb2	6.21 ± 0.52 ^{a}	0.95 ± 0.08 ^{c}	**41.71 ± 1.80** ^{e}	5.27 ± 0.49 ^{c}
	Gb11	**3.79 ± 0.40** ^{b}	0.61 ± 0.13 ^{c}	5.55 ± 0.53 ^{b}	9.89 ± 0.61 ^{d}
	Gb15	**4.14 ± 0.24** ^{b}	0.55 ± 0.14 ^{c}	3.66 ± 0.54 ^{f}	**13.03±0.29** ^{e}

*Values reported are the means ± standard deviations (n = 5, 95 % confidence interval). Different superscript letters in the same column indicate a statistically significant difference (Tukey test $p < 0.05$). Shadowed in bold are the best values.

The opacity means a smaller transparency, important to control the incidence of light on the cheese (Cuq et al., 1996). Opacity values increase with the concentration in polysaccharide for films from solutions of *Gb*, being the solutions with sorbitol and oil those with a higher value of opacity. The addition of lipid caused the films to become whitish. Table 4.2 shows that the incorporation of corn oil in the films increased the opacity. Yang and Paulson, (2000) demonstrated that also gellan film has increased opacity with the increase of lipid concentration.

4.4 Conclusion

This work shows how the wettability (*Ws*) can be used as a parameter for coating optimization. The cheese surfaces were found to be of low-energy and therefore Zisman's method was used to determine their wettability. Cheese has the ability to participate in non-polar interactions, as a consequence of the higher values of the dispersive component. The best values in terms of *Ws* were obtained for cheese with the following formulations, 0.5 % of polysaccharides of *G. birdiae*, 2.0 % of glycerol; 1.5 % of polysaccharides of *G. birdiae*, 0.5 % of glycerol and 0.5 % of oil; and 1.5 % of polysaccharides of *G. birdiae*, 0.5 % of glycerol/sorbitol and 0.5 % of oil. This procedure is important in order to ensure that the application of the coating solutions on the cheese is made uniformly and easily, in view of future industrial uses. These coatings showed lower values of O_2 permeability, which are important once the oxygen in contact with the cheese contributes to lipid oxidation and to the growth of undesirable microorganisms.

In terms of *WVP* the Gb11 solution showed lower values of *WVP*. This characteristic is important in the maintenance of water content, therefore reducing cheese weight loss throughout storage, and suggests an improved efficiency in water loss control, thus improving cheese quality attributes and extending its shelf life. Decreasing the light incidence on the cheese (light promotes fat oxidation) is another important achievement. The solution Gb15 showed interesting high values of opacity.

These findings provided important information on properties of *Gb* agar films in view of their use by the food industry e.g., as coatings and films for the improvement of food storage conditions.

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Chapter 5 - Effect of moderate electric fields in the permeation properties of chitosan coatings

5.1 Introduction

Edible coatings can provide an alternative to extend the post-harvest life of fresh fruits and other vegetables and can also result in a similar effect as modified atmosphere storage in modifying the internal gas composition (Park, 1999). Indeed, this protective barrier can be formulated to prevent the transfer of moisture, gases, flavor or lipids, and thus to maintain or improve food quality and to increase food product shelf life (Krochta and De Mulder-Johnson, 1997). Carbohydrates (starches, polysaccharides), proteins, lipids, and combinations of these can be used to make edible films. Chitosan is a chitin-derived polysaccharide and is one of the most abundant natural polymers, largely widespread in living organisms such as shellfish, insects, and mushrooms. It is a polysaccharide with linear structure constituted by a copolymer of β-(1-4)-linked D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc) residues (Tharanathan and Kittur, 2003). It is obtained chiefly by homogeneous deacetylation of chitin with strong bases, rendering chitosans of different acetyl content or deacetylation degrees. Chitosan is a versatile biopolymer, having a broad range of applications in the food industry (Rudrapatnam and Farooqahmed, 2003). The performance of edible coatings depends on their composition and the conditions in which they are used (e.g. relative humidity). A plasticizer is generally required for edible films to overcome film brittleness. Plasticizers could reduce the intermolecular forces and increase the mobility of polymer chains, therefore improving the flexibility and extensibility of the films. Nevertheless, the addition of plasticizers also increases (in general) gas and water vapor permeability of the film, and could possibly decrease the mechanical strength (Gontard et al., 1994; Mali et al., 2004).

Ohmic heating is based on the passage of electrical current through a sample that has electrical resistance. The electrical energy is directly converted to heat and instant heating occurs, at a rate, which depends on the intensity of the current passing through the material. There are practically no works dealing with the subject of producing edible films under an electric field;(Lei et al., 2007), wrote one of the very few, where they reported that ohmic heating had many advantages in the production of protein-lipid film, including the improvement of the yield, film formation rate and rehydration capacity of protein-lipid films.

Atomic Force Microscopy (AFM) is one of the techniques that has been used to characterize the surface microstructure e.g. of plasticized soy protein isolate films (Ogale et al., 2000).

AFM imaging modes can potentially provide structural information for a sample in its more natural state (without dehydration or coatings) (Lent et al., 1998). Nanoscale measurements by AFM allow the influence of different factors on the hardness, elasticity and permeability of the film surface to be quantified, which is extremely useful for the design of high-performance edible food packaging systems (Herrmann et al., 2004). Measurements of the topography and roughness can be undertaken with extremely high resolution. This technique has been used to characterize the surface morphology of whey protein films (Herrmann et al., 2004; Lent et al., 1998).

The aim of this work was to study the effect of field strength on transport properties of chitosan coatings and film structure, therefore providing insight on the effect of the electric fields on films structure.

5.2 Materials and methods

5.2.1 Coating Materials

The materials used to prepare the edible coating solutions were: chitosan (obtained in the Pharmaceutical Laboratories Mario Muñoz, Cuba) with a degree of deacetylation of 90 % approximately, Tween 80 (Acros Organics, Belgium) as surfactant and lactic acid (Merck, Germany).

5.2.2 Film Formation

The coating solutions were prepared dissolving the chitosan (1.5 % w/v) in a 1 % (v/v) lactic acid solution with agitation using a magnetic stirrer during 2 hours at room temperature (20 °C); subsequently, Tween 80 was added as a surfactant at a concentration of 0.1 % (w/w) (Casariego et al., 2008). After homogenizing, the chitosan solution was filtered to remove most of the undissolved impurities (< 1 % of the chitosan content). At the end of these treatments, a constant amount (28 mL) of chitosan solution was cast onto an 8 cm diameter glass plate in order to maintain the film thickness. The films were dried in an oven at 35 °C during 8 hours. Dried films were peeled from the plate and cut in circles with 8 cm of diameter, approximately, for property testing.

5.2.3 Device description

A set of experiments was conducted to determine the effect of the application of a moderate electric field to chitosan solutions. The chitosan solution samples were treated in an ohmic heater using four different field strengths (from 50 to 200 Vcm^{-1}) with a 2 cm gap between the electrodes, in all cases leading to an increase of temperature up to 60 °C. The heater and data acquisition system used are represented in Figure 5.1 and consisted of a cylindrical glass tube of 30 cm total length and 2.3 cm inside diameter; two Titanium electrodes with Teflon pressure caps were placed at each end of the tube. Samples were heated using an alternating current source of 50 Hz, with different field strengths. Temperatures were monitored using a type-K thermocouple, placed at the geometrical centre of the chamber though the available opening. A data-logger was employed to record continuously and simultaneously, current intensity, voltage and temperature. In order to measure voltage across and current through the samples voltage and current transducers were used, respectively.

In order to collect data for the conventional heating, 30 mL Falcon tubes containing the chitosan solution samples were placed in a temperature controlled water bath. The thermal history of the samples, until temperature stabilization, was monitored by the introduction of a thermocouple connected to the data acquisition system previously described; this treatment was used as control in order to discard the temperature effects and to evaluate only the effects of electric field.

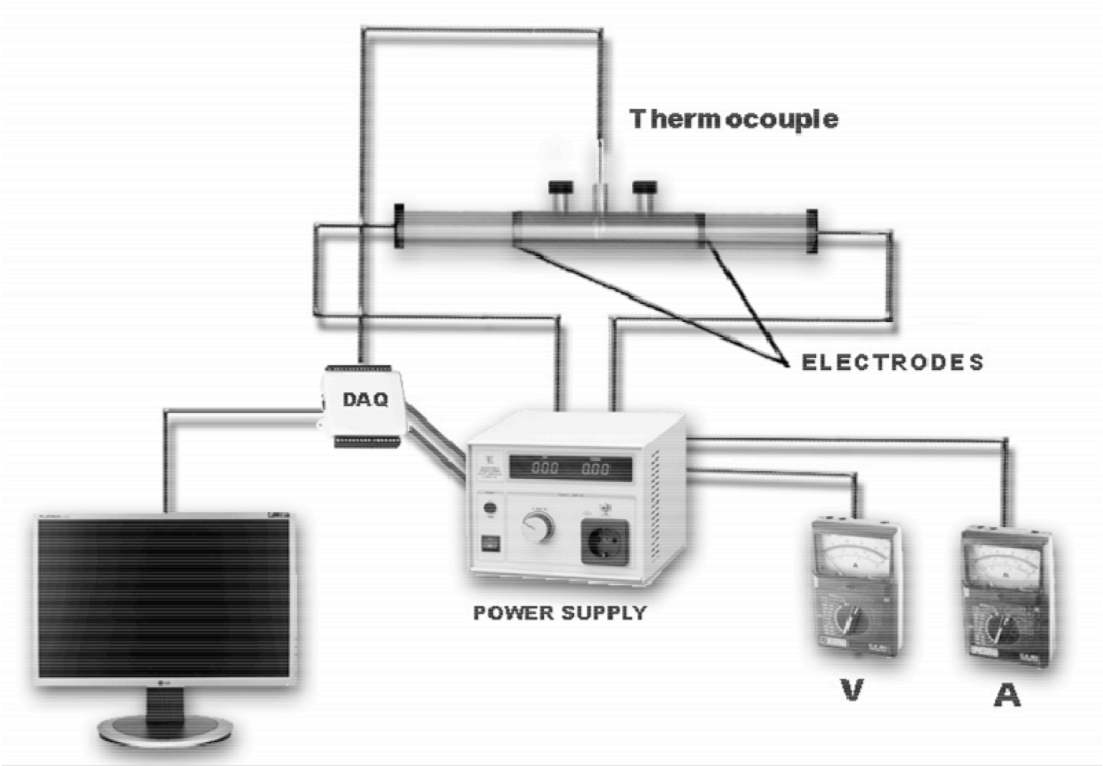

Figure 5.1 - Schematic diagram showing the device used to perform ohmic heating. DAQ = Data Acquisition. V= Voltage. A= Amperage.

5.2.4. Characterization of chitosan films

5.2.4.1 Conditioning

All chitosan films used for permeability tests were conditioned in desiccators, at 20 °C and 25 % RH.

5.2.4.2 Thickness

Film thickness was measured with a hand-held digital micrometer (Mitutoyo, Japan) having a sensitivity of 0.001 mm. Ten thickness measurements were taken on each testing sample in different randomly chosen points and the mean values were used in permeability calculations.

5.2.4.3 Optical properties

The color of films was determined with a Minolta colorimeter (CR 400; Minolta, Japan). A white color plate (*Y*=93.5, *x*=0.3114, *y*=0.3190) was used as standard for calibration. The CIELab scale was used to measure lightness (*L*) and chromaticity parameters *a** (red – green) and *b** (yellow – blue). Measurements were performed placing the film sample over the standard. Samples were analyzed in triplicate, recording four measurements for each sample.

The opacity of a material is an indication of how much light passes through it. The higher the opacity, the lower the amount of light that can pass through the material. Generally, opacity is calculated from reflectance measurements. The opacity of the samples was determined, according to Hunter lab method, as the relationship between the opacity of each sample on a black standard (Y_b) and the opacity of each sample on the white standard (Y_w):

$$Opacity = \frac{Y_b}{Y_w} \times 100\% \qquad \text{Eq 5.1}$$

Where *Y* is the CIE tristimulus value.

5.2.4.4 Permeability of gases

Oxygen permeability (O_2P) and carbon dioxide permeability (CO_2P) were determined based on the (ASTM-D-3985-02, 2002) method. A chitosan film was sealed between two chambers, having each one two channels to the exterior. In the lower chamber O_2 or CO_2 were supplied at a controlled flow rate to keep the pressure constant in that compartment. The upper chamber was purged by a stream of nitrogen, also at a controlled flow. This nitrogen acted as a carrier for the O_2 or CO_2 coming from the lower chamber though the film. The flows of the two chambers were connected to manometers to ensure the equality of pressures between both compartments, kept at 1 atm. As the O_2 or CO_2 were carried continuously by the nitrogen flow, it was considered that O_2 or CO_2 partial pressure in the upper compartments is null, therefore ΔP can be considered to be 1 atm. O_2P was determined from the measurements of O_2 concentration in the nitrogen flow leaving the chamber with an O_2 sensor installed on-line.

CO_2P was determined from the measurements of CO_2 concentration in a gas chromatograph (Chrompack 9001, Middelburg, Netherlands) with a column Porapak Q 80/ 100 mesh- 2 m x 1/8 "x 2 mm SS (Temperatures: Oven = 35 °C, detector and injector = 110 °C; flow of the carrier gas = 23 $mL \cdot min^{-1}$). In all cases the variation coefficient obtained between the three replicates made for each experiment was below 5 %.

5.2.4.5 Water vapor permeability measurement

The water vapor permeability (*WVP*) of the films was determined gravimetrically based on the ASTM E96-92 method (Guillard et al., 2003; McHugh et al., 1993). The test film was sealed on the top of a permeation cell containing distilled water (100 % RH; 2.337×10^3 Pa vapor pressure at 20 °C), placed in a desiccator which was maintained at 20 °C and 0 % RH (0 Pa water vapor pressure) with silica gel. The water transferred through the film and adsorbed by the desiccant was determined from weight loss of the permeation cell. The cups were weighed at intervals of 2 hours during 10 hours. Steady-state and uniform water pressure conditions were assumed by keeping the air circulation constant outside the test cup by means of a miniature fan placed inside the desiccators (McHugh et al., 1993). The slope of the curve representing the weight loss versus time was obtained by linear regression. The measured (*WVP*) of the films was determined as follows:

$$WVP = (WVTR.L) / \Delta P \qquad \text{Eq. 5.2}$$

Where *WVTR* is the measured water vapor transmission rate ($g \cdot m^{-2} \cdot s^{-1}$) through the film (calculated from the slope of the curve divided by the area of the film), *L* is the mean film thickness (m), and ΔP is the partial water vapor pressure difference (Pa) across the two sides of the film. For each type of film, *WVP* measurements were replicated three times and the variation coefficient obtained was at all times below 5 %.

5.2.4.6 Film solubility

The film solubility in water was determined according to the method reported by (Gontard et al., 1994). It was defined by the content of dry matter solubilized after 24 h immersion in water.

The initial dry matter content of each film was determined by drying it to constant weight in an oven at 105 °C. Two disks of film (2 cm diameter) were cut, weighed, and immersed in 50 mL of water. After 24 h of immersion at 20 °C with occasional agitation, the pieces of film were taken out and dried to constant weight in an oven at 105 °C, to determine the weight of dry matter, which was not solubilized in water. The variation coefficient obtained between the three replicates made for each experiment was below 5 %.

5.2.4.7 Atomic force microscopy

The surface morphology of the films was analyzed by AFM with a Nanoscope III, Multimode (Digital Instruments) with a 10 μm x 10 μm scan size and a 3.5 μm vertical range. Measurements were taken from several areas of the film surface (10 μm·10 μm) using the tapping mode. The resulting data set for each sample was transformed into a 3D image. The average sample roughness (ASME, 1995), (*Ra*) was estimated with the aid of the built-in software of the equipment.

5.2.4.8 Statistical analysis

Analysis of Variance (Olivas and Barbosa-Cánovas) and linear regression were the main statistical tools used for data analysis. The Tukey test (α = 0.05) was also used to determine the significance of differences between specific means (SigmaStat 3.1, 2004, Excel, 2003, USA).

5.3 Results and discussion

5.3.1 Oxygen permeability (O_2P) and Carbon dioxide permeability (CO_2P)

Permeability is a steady-state property that describes the extent to which a permeating substance dissolves and then the rate at which it diffuses through a film, with a driving force related to the difference in concentration of that substance between the two sides of the film (Gennadios et al., 1993).

Gas permeability of edible films and coatings depend on several factors such as the integrity of the film, the ratio between crystalline and amorphous zones, the hydrophilic-hydrophobic ratio and the polymeric chain mobility; the interaction between the film-forming polymer and the presence of a plasticizer or other additives are also important factors in film permeability (Garcia et al., 2000). The measurement of

the permeability of edible films to oxygen and carbon dioxide provides important information for the development of edible films. Oxygen is the key factor that might cause oxidation, inducing several unwanted food changes such as odor, color and flavor, as well as nutrients deterioration. Therefore, films providing a proper oxygen barrier can help improving food quality and extending food shelf life (Sothornvit and Pitak, 2007). Carbon dioxide is formed in some foods due to deterioration and respiration reactions. The produced CO_2 has to be removed from the package to avoid food deterioration and/or package destruction (Vermeiren et al., 2003). Such films can maintain food quality and improve stability and shelf life by retarding unwanted mass transfer in food products (Miller and Krochta, 1997), including to retard transport of gases (O_2, CO_2) for fruits and vegetables, migration of moisture for dried and intermediate moisture foods, and migration of solutes for frozen foods. Figure 5.2 shows O_2P and CO_2P as measured for chitosan films formed from solutions subjected to electric fields of different intensities. The samples with treatments made at 100 $V \cdot cm^{-1}$ or higher have lower values ($p < 0.05$) of O_2P and CO_2P.

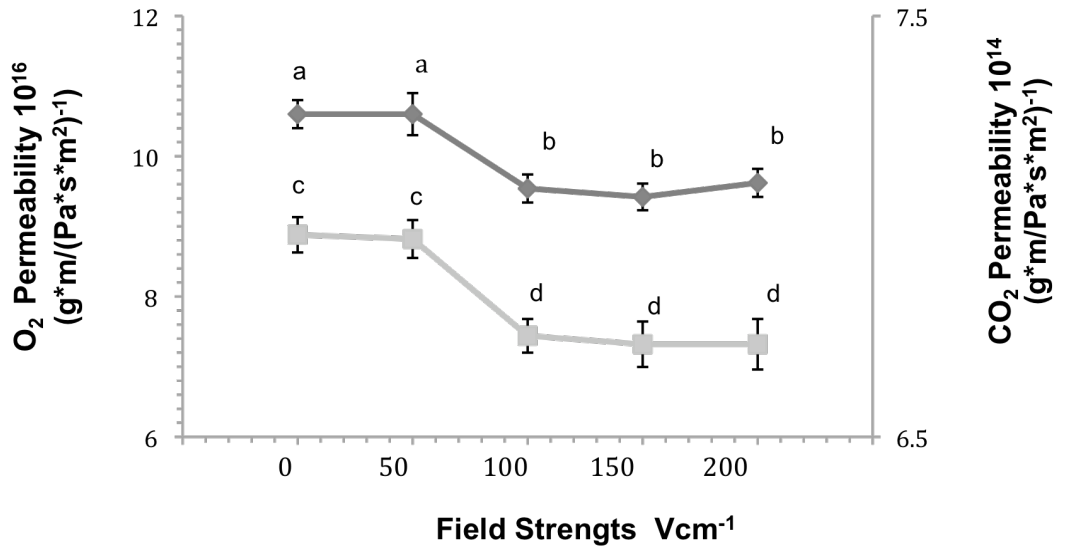

Figure 5.2 - O_2 permeability (♦) and CO_2 permeability (■) of chitosan films. Different letters in the data points correspond to statistically different samples ($p < 0.05$).

The *AFM* observation of a regular surface of the chitosan films treated at 100 V·cm^{-1} or above as opposed to a rougher surface of the untreated films indicates that the films structure might have been altered due to the application of the electric field during the preparation of the film-forming solution (Figure 5.3). (Wan et al., 2003) observed that the crystallinity of the chitosan membranes increased gradually with increasing degree of deacetylation ranging from 70 to 90 %. This can be attributed to the fact that chains of chitosan with higher degree of deacetylation are more compact thus facilitating hydrogen-bonding formation and consequently favoring crystallinity formation in the film. Furthermore, chitosan with a higher degree of deacetylation contains more glucosamine groups, which also facilitate the hydrogen-bonding formation; on the contrary, chitosan with a lower degree of deacetylation has more acetyl groups, which hinder the chitosan chain packing due to their rigidity and steric effect (Bangyekan et al., 2006). (Lei et al., 2007), studied the effects of different heating methods on the production of protein-lipid film and concluded that the major advantage of ohmic heating is that the heat is dispersed uniformly throughout the whole liquid compared to water bath heating, and finally concluded that the film formation rate was higher when ohmic heating was applied. During the heating process, heat was uniformly applied to the whole volume of the film, accelerating the collisions between molecules. This process can provide an improvement in the crystallinity of the chitosan film, thus increasing the material´s resistance to gas permeation. (Balau et al., 2004) studied the X-ray difractogram of chitosan films, an almost amorphous structure; the films treated with an electric field of E = 20 kV·cm^{-1}, developed a crystalline structure, while the films to which no electric field was applied displayed a significantly lower proportion of crystalline material, showing that the electric field plays an important role in the crystallization process.

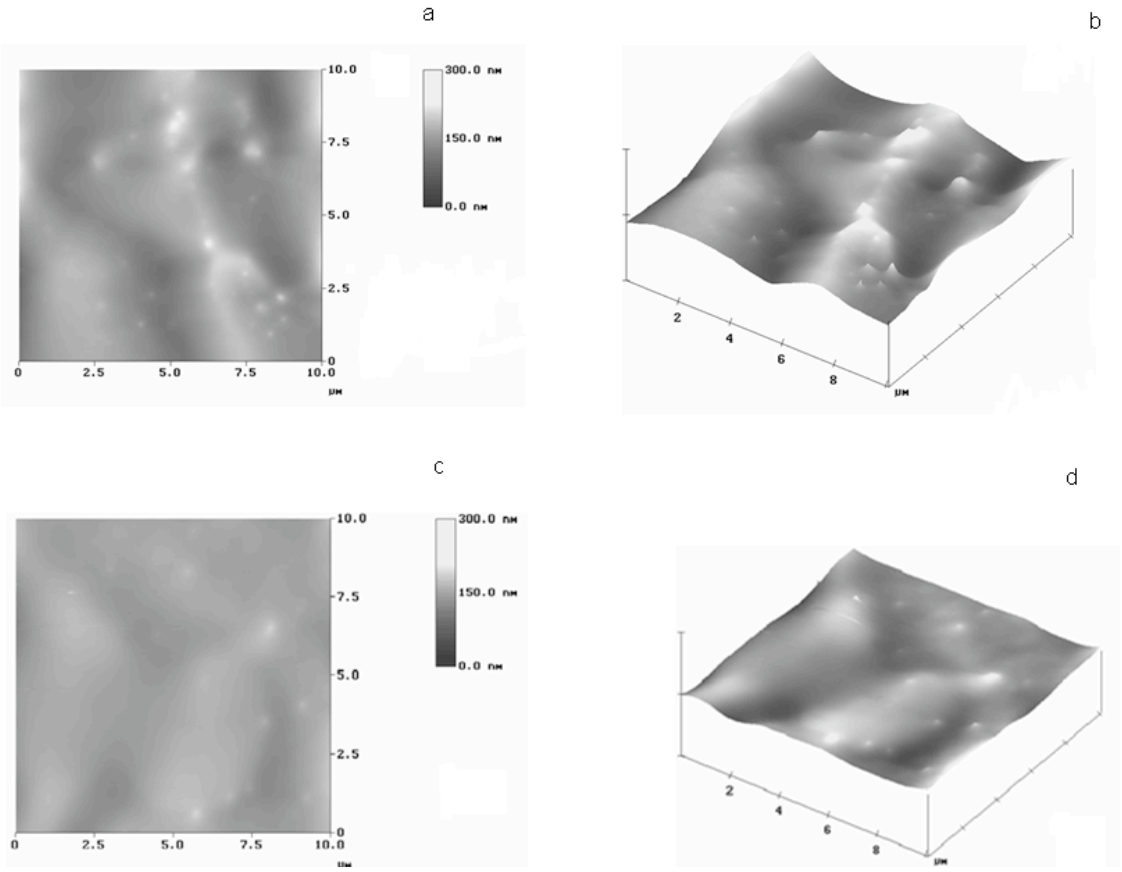

Figure 5.3 - AFM topographic images of (a) chitosan films obtained from film-formed solutions treated at a field strength of 50 Vcm^{-1} (b) is three-dimensional image. (c) AFM topographic images of chitosan films obtained from film-formed solution treated at field strength of 100 Vcm^{-1} (d) is three-dimensional image.

5.3.2 Water vapor permeability

Water vapor permeability (*WVP*) is an important parameter commonly considered in food packaging. *WVP* comprises sorption, diffusion and adsorption and is largely governed by the interactions between the polymer and the water molecules (Nivedita et al., 2004) . Water permeation through a film usually occurs through the hydrophilic part of the film, thus the relation of the hydrophilic/hydrophobic portions is important to determine *WVP*. Polymers with high hydrogen bonding produce films that are susceptible to moisture while polymers with hydrophobic groups make excellent barrier to moisture. Generally, *WVP* is also dependent on the pore size of the film (Paramawati et al., 2003) .

In fact, *WVP* tends to increase with polarity, degree of unsaturation and degree of ramification of the lipids used (if any), in addition to the effect of the water molecule sorption by the polar part of the film material (Gontard et al., 1994).

Butler et al. (1996) reported that chitosan films are highly impermeable to oxygen, however they have relatively poor water vapor barrier characteristics, which result from their hydrophilicity.

The water vapor permeability should be as low as possible since an edible film or coating should retard moisture transfer between the food and the environment, or between two components of a heterogeneous food product (Gontard et al., 1992).

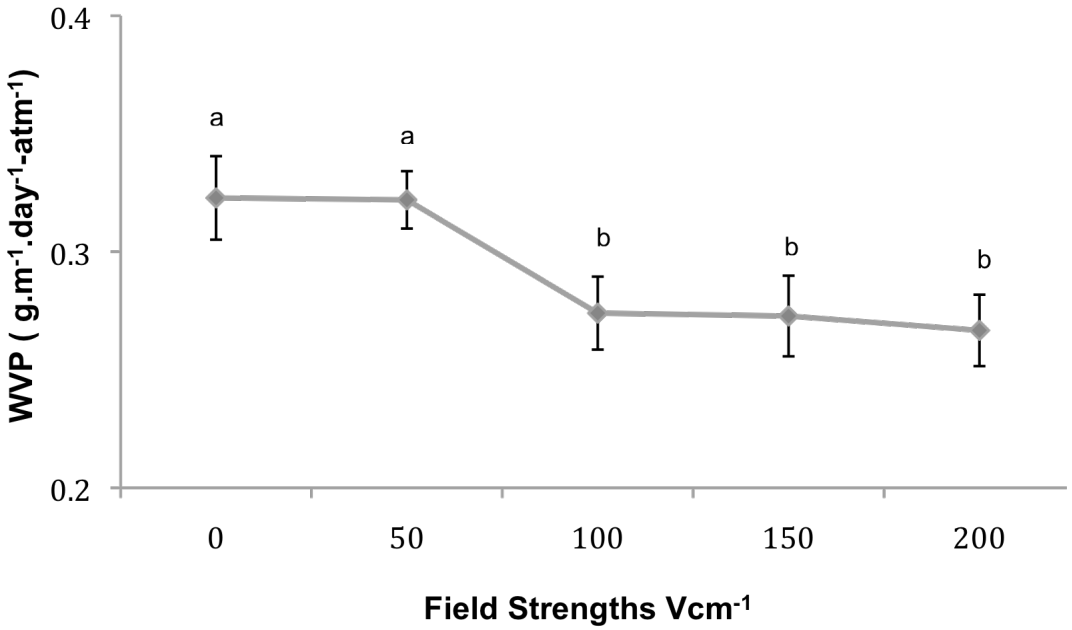

Figure 5.4 - Variation of water vapor permeability for different field strengths. Different letters in the data points correspond to statistically different samples ($p < 0.05$).

The results obtained in this work show that *WVP* of chitosan films decrease (up to 17.3 %) with the increase of the field strengths for values of 100 V·cm^{-1} or higher. These films showed lower *WVP* values than those of other hydrocolloids films reported in literature (Bravin et al., 2006; Bravin, 2006; Olivas and Barbosa-Cánovas, 2008; Vargas et al., 2009; Ziani et al., 2008).

The increase of the field strength seems to be correlated with the decrease of permeability (see Figure 5.4). Herrmann et al. (2004) observed that an increase of protein concentration lead to an increase of the viscosity of the film-forming solution, which resulted in the incorporation of air bubbles; this formed non-homogeneous and non-compact film networks, increasing the roughness and, as a consequence, the value of *WVP*.

Anker et al. (2000) concluded that the reason for the increased *WVP* is probably the larger pores formed at high polymer concentration, compared to the smaller pores formed at low polymer concentration. The work of Miller and Krochta, (1997), also points at the fact that the permeability is highly affected by how closely packed the polymer chains are, thus establishing a direct relationship between the crystallinity of the structure and permeability.

5.3.3. Solubility in water and optical properties

5.3.3.1 Solubility in water

Solubility in water is defined as the maximum percentage (by weight) of a substance that will dissolve in a unit volume of water at certain (usually room) temperature. It is an important property, which governs potential applications of these materials to food preservation. Films with low water solubility are necessary for the protection of foodstuffs with high or intermediate water activity (Sébastien et al., 2006) . On the other hand, edible films with high water solubility may be required, for example, to contain premeasured portions which will be dissolved in water or in hot food (Guilbert and Biquet, 1989).

In the present work the solubility of the chitosan films was evaluated, and it is shown that the solubility of chitosan films decreases with the increase of the field strength for values of 100 $V \cdot cm^{-1}$ or higher (Figure 5.5).

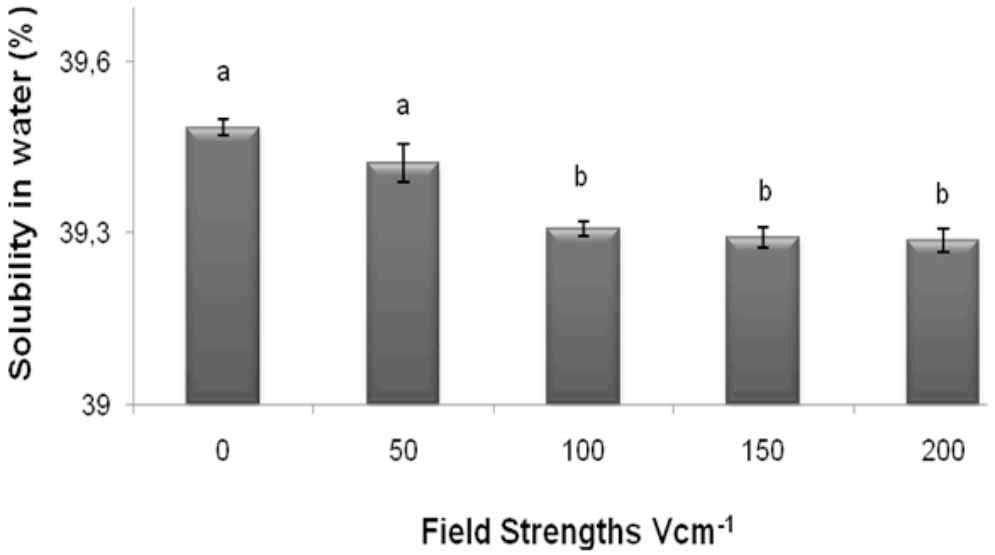

Figure 5.5 - Solubility in water of chitosan films treated different electrical field strengths. Different letters in the same column correspond to statistically different samples ($p < 0.05$).

Balau et al. (2004) showed that the electric field plays an important role in the crystallization process, which may also interfere in the water solubility of the films. In fact, similarly to what has been reported for gas permeability, the solubility of chitosan films has been associated to the crystallinity of the sample; a high crystallinity contributes to a higher insolubility (Du and Hsieh, 2007), and the poor solubility of chitosan has been attributed to its partially crystalline structure (Nishimura et al., 1991).

5.3.3.2 Optical properties

The results of the measurements of color are shown in Table 5.1. The films should be visually attractive, and should not change their color throughout the time of storage, in order not to harm the acceptance of the product on which they are applied.

The results show that the lightness of chitosan films is somewhat lower when an electric field is applied, but still high. For comparison, results for the lightness (L^*) of albumen (from egg) range between 95.67 and 96.20 (Gennadios et al., 1996); such films were reported to be clearer and more transparent than films based on wheat, soy protein and corn zein, studied by the same authors.

Further, the values of lightness for chitosan films are higher than those reported for wheat protein films, which presented values of L^* between 83.3 and 89.7 (Rayas et al., 1997). The high values of the component b^* indicate the predominance of the yellow color in the chitosan films; this coincides with the data reported by (Butler et al., 1996). Our results also indicate that an increase in the field strength leads to a significant increase of the values of b^* (see Table 5.1). In general, polysaccharide films are free from the color problems associated with protein (which can suffer Maillard reactions) and lipid (which can suffer oxidation) films (Trezza and Krochta, 2000).

Table 5.1 - Optical properties of chitosan films

Electric field strenght	L^* (lightness)	a*	b*	Opacity (%)
0 Vcm^{-1}	93.80 ± 0.38[a]	4.04± 0.11[a]	11.01 ± 0.46[a]	4.98 ± 1.11[a]
50 Vcm^{-1}	94.01 ± 0.72[a]	4.15± 0.17[a]	11.64 ± 0.56[a]	5.05 ± 0.97[a]
100 Vcm^{-1}	93.68 ± 0.56[a]	4.29± 0.27[a]	20.73 ± 1.57[b]	5.07 ± 0.28[a]
150 Vcm^{-1}	93.73 ± 0.91[a]	4.50± 0.40[a]	21.26 ± 1.64[b]	5.06 ± 0.61[a]
200 Vcm^{-1}	93.84 ± 0.98[a]	4.00± 0.47[a]	20.34 ± 1.46[b]	4.89 ± 0.47[a]

*Different letters in the same column correspond to statistically different samples ($p < 0.05$).

The evaluation of the opacity of a material demonstrates its greater or lesser transparency. For the development of materials meant to be used as films or coatings for food, increased transparency tends to be better (Yang and Paulson, 2000), once the goal is to retain the original features of the product, such as color.

The values of opacity (Table 5.1) for the films under consideration did not differ significantly ($p < 0.05$); all films were transparent, meaning that there was no apparent effect due to the application of an electrical field.

5.4 Conclusions

The results obtained showed that the application of a moderate electric field to the film-forming solutions has statistically significant effects on the film´s physical properties and structure. In general, the most pronounced effect of the field strength was observed for treatments made at 100 $V \cdot cm^{-1}$ or higher. The solubility in water and the water vapour, oxygen and carbon dioxide permeability coefficients showed a positive correlation with the application of an electric field. In practice, the changes in the film properties induced by the application of the electrical field may translate into an improved shelf-life of the products due to reduced water loss (calculated on the basis of the lower *WVP* values achieved) and reduced O_2 and CO_2 exchanges (due to the lower values of O_2P and CO_2P), which will mean a slower metabolism e.g. in fruits and vegetables (Casariego et al., 2008). Future work should be directed towards the confirmation of these effects in real food systems.

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Chapter 6 - Influence of electric fields in the structure of chitosan edible coatings

6.1 Introduction

Biodegradable and renewable materials are an important part of the effort to reduce the impact of food packaging in the environment. The development of edible films or coatings based on natural biopolymers (proteins, polysaccharides and their derivatives) provides a potential alternative to non-biodegradable packaging materials. Chitosan shows wide application potentials in biology, medicine, food, electrochemistry, and membrane separation due to its attractive characteristics of low price, biocompatibility, hydrophilicity and chemical versatility (Liu et al., 2009). Chitosan has been widely used for the production of edible coatings and edible films (Casariego et al., 2009; Casariego et al., 2008; Jeon et al., 2002). Chitosan films are excellent oxygen and carbon dioxide barriers and have interesting antimicrobial properties (Casariego et al., 2009; Dutta et al., 2009).

The cationic character of chitosan offers good opportunities to take advantage of electron interactions with numerous compounds during processing and to incorporate specific properties into the material (Lacroix and Le Thien, 2005).

The use of electric fields in the food area has gained a new interest in recent years (Castro et al., 2003; Castro et al., 2004; Icier and Ilicali, 2005). The application of electric fields has also been an important instrument among researchers in the area of edible films and coatings, and there are works showing that the application of electric fields promotes a significant improvement of several properties (García et al., 2009; Lei et al., 2007; Souza et al., 2009).

Lei et al. (2007) reported advantages in the use of ohmic heating for the production of protein-lipid films, including the improvement of the yield, of the film formation rate and of the rehydration capacity of the films. García et al. (2009) analyzed the effect of applying an electrical field during drying on the microstructure of films formulated with different concentrations of chitosan and methyl-cellulose; those authors have shown that the electrical field treatment could be a good alternative to improve film flexibility and to increase water vapor barrier properties. Souza et al. (2009) applied a moderate electric field to chitosan film-forming solutions and showed that it affects the physical properties and structure of the films and coatings which are then reflected on their transport properties.

In this context, the objectives of the present work were to analyze the effect of applying a moderate electric field to film-forming solutions of chitosan, to evaluate the films' microstructure by SEM and X-ray diffraction analyses and to analyze their thermal and mechanical properties.

6.2. Materials and methods

6.2.1 Coating materials

The materials used to prepare the edible coating solutions were: chitosan (obtained in the Pharmaceutical Laboratories Mario Muñoz, Cuba) with a degree of deacetylation of 90 % approximately, Tween 80 (Acros Organics, Belgium) as surfactant and lactic acid (90 %, Merck, Germany).

6.2.2 Film formation

The coating solutions were prepared dissolving the chitosan (1.5 % w/v) in a 1 % (v/v) lactic acid solution with agitation using a magnetic stirrer during 2 hours at room temperature (20 °C); subsequently, Tween 80 was added as a surfactant at a concentration of 0.1 % (w/w) (Casariego et al., 2008). After homogenizing, the chitosan solution was filtered to remove most of the undissolved impurities (< 1 % of the chitosan content). At the end of these treatments, a constant amount (28 mL) of chitosan solution was cast onto an 8 cm diameter glass plate in order to maintain the film thickness. The films were dried in an oven at 35 °C during 8 h. Dried films were peeled from the plate and cut in circles with 8 cm of diameter, approximately, for property testing.

6.2.3 Device description

A set of experiments was conducted to determine the effect of the application of a moderate electric field to chitosan solutions. The chitosan solution samples were treated in an ohmic heater using different field strengths (from 100 and 200 V cm^{-1}) with a 2 cm gap between the electrodes, in all cases leading to an increase of temperature up to 60 °C (for details of the apparatus please see section 5.2.3, chapter 5). In order to collect data for the conventional heating, 30 mL Falcon tubes containing the chitosan solution samples were placed in a temperature controlled water bath. The thermal history of the samples, until temperature stabilization, was monitored by the introduction of a thermocouple in the geometrical center of the tubes, connected to the data acquisition system previously described; this treatment was used as control in order to discard the temperature effects and to evaluate only the effects of the electric field.

6.2.4 Characterization of chitosan films

6.2.4.1 Conditioning

All chitosan films used for permeability tests were conditioned in desiccators, at 20 °C and 25 % RH

6.2.4.2 Thickness

Film thickness was measured with a hand-held digital micrometer (Mitutoyo, Japan) having a sensitivity of 0.001 mm. Ten thickness measurements were taken on each testing sample in different randomly chosen points and the mean values were used to calculate films properties.

6.2.4.3 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) analyses were performed with a scanning electron microscope (Nova NanoSEM 200, The Netherlands) with an accelerating voltage varying from 10 to 15 kV.

6.2.4.4 X-ray diffraction and Crystallinity

X- ray diffraction patterns of the films were analyzed between $2\theta = 4°$ and $2\theta = 60°$ with a step size $2\theta = 0.02°$ in an X- ray diffraction instrument (Bruker D8 Discover, USA). The crystallinity index (CI) was defined using the equation $CI = (I_{110} - I_{am})/I_{110}$ (Srinivasa, Ramesh, Kumar, & Tharanathan, 2004), where I_{110} is the maximum intensity (2θ, 20°) of the (110) lattice diffraction and I_{am} is the intensity of the amorphous diffraction (2θ, 16°).

6.2.4.5 Mechanical properties: tensile strength (TS) and elongation-at-break (E)

TS and *E* were measured with an Instron Universal Testing Machine (Model 4500, Instron Corporation, USA) following the guidelines of the ASTM Standard Method D 882-91. The initial grip separation was set at 30 mm and the crosshead speed was set at 5 mm/min. *TS* was expressed in MPa and calculated by dividing the maximum load (N) by the initial cross-sectional area (m^2) of the specimen. *E* was calculated as the ratio of the increased length to the initial length of a specimen (30 mm) and expressed as a percentage. *TS* and *E* tests were replicated five times for each type of film.

6.2.4.6 Differential scanning calorimetry (DSC)

DSC measurements were performed with a Shimadzu DSC-50 calorimeter (Shimadzu Corporation, Kyoto, Japan). About 10 mg of the samples were placed in Aluminium DSC pans. The samples were heated from 25 to 350 °C at a heating rate of 10 °C min^{-1} under a Helium atmosphere.

6.2.4.7 Statistical analyses

Analysis of variance and linear regression were the main statistical tools used for data analysis. The Tukey test (α = 0.05) was also used to determine the significance of differences between specific means (SigmaStat 3.1, 2004, Excel, 2003, USA).

6.3 Results and discussion

6.3.1 X-ray diffraction

Chitosan can exist in two distinct crystal forms, and many different patterns observed by X-ray diffraction represent different mixtures of the two forms. The use of higher molecular-weight (*MW*) chitosan produces a less crystalline, smaller crystallite, rod structured film, while chitosan with lower *MW* forms a more crystalline, and a larger crystallite spherulitic film. In summary, chitosan and chitosan-derived networks usually exhibit a semi-crystalline structure due to the free-energy balance caused by hydrogen bonding formation (Costa-Júnior et al., 2009).

The X-ray diffraction patterns of all film samples are displayed in Figure 6.1. The diffraction peak around 19°-20° (2θ) observed for chitosan films in this work is in agreement with other published results (Bangyekan et al., 2006; Srinivasa et al., 2004; Ziani et al., 2008).

Figure 6.1 shows that the crystallinity of chitosan films increases gradually with the increase of the electric field strength. This indicates that, during the moderate electric field treatment, a structure with a different X-ray diffraction pattern was developed. This may be attributed to the fact that the chitosan chains with higher degree of deacetylation are more flexible. Flexible chains will facilitate the hydrogen bond formation and consequently crystallinity formation in the film. In addition, there was one other diffraction peak at around 8°-10° (2θ).

Zhang et al. (2006) have shown that the reflection around 10° (2θ) reflects the presence of a crystal form I and the strongest reflection at 2θ = 20° corresponds to a crystal form II.

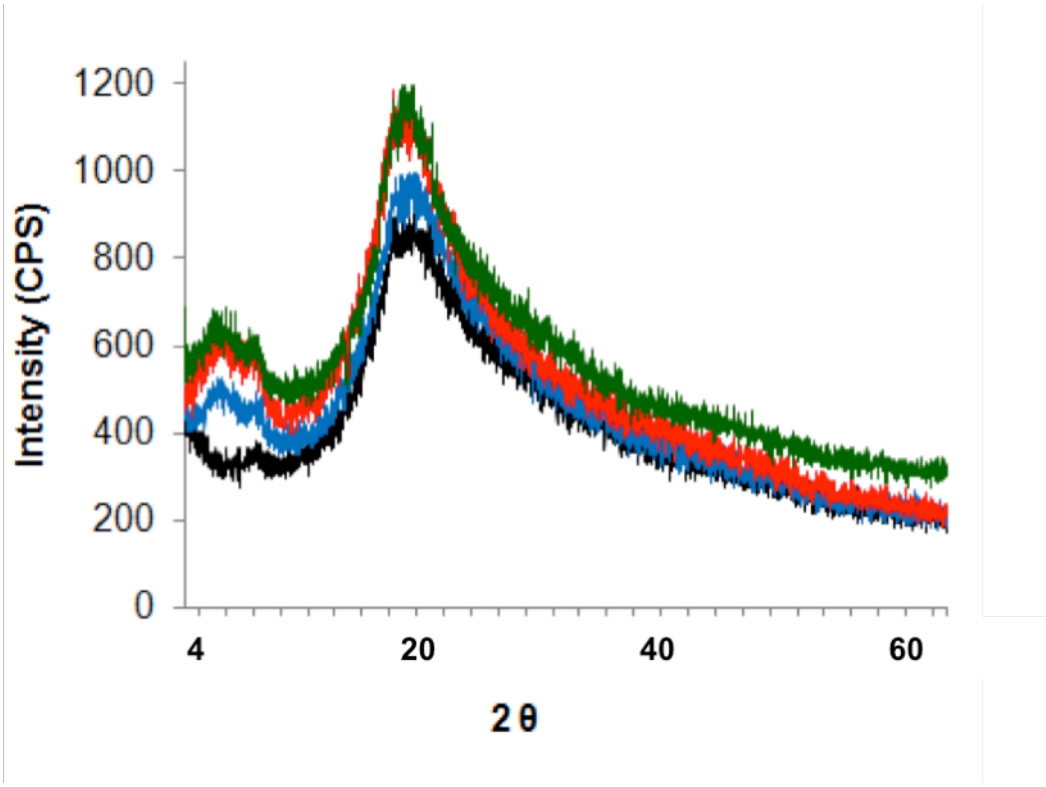


Figure 6.1 - X-ray diffraction patterns of chitosan films: (-) Control (no heating); (-) Conventional heating (-); Electric fields at 100 V cm $^{-1}$; and (-) Electric fields at 200 V cm^{-1}.

Wan et al. (2006) demonstrated that the X-ray pattern of the chitosan membrane has two characteristic peaks located at 2θ of about 10.2° and 20.2°. It is known that chitosan always contains bound water (5 %) even if it has been extensively dried. The incorporation of bound water molecules into the crystal lattice, commonly termed hydrated crystals, generally gives rise to a more dominated polymorph, which can be normally detected by a broad crystalline peak in the corresponding X-ray pattern. Therefore, the crystalline peak centered at around 10° is attributed to the hydrated crystalline structure of chitosan (Ogawa et al., 1992; Wan et al., 2006).

Another peak registered near 20° is reported to be the indication of the relatively regular crystal lattice (110) of chitosan (Wan et al., 2006; Yamamoto et al., 1997).

Results indicated that the application of a moderate electric field to the film-forming solutions had significant effects on the crystallinity index (CI), which was higher for films treated with electrical fields.

Balau et al. (2004) studied the X-ray difractograms of chitosan films, an almost amorphous structure. They showed that films treated with an electric field of E = 20 kV·cm^{-1}, developed a crystalline structure, while the films to which no electric field was applied displayed a significantly lower proportion of crystalline material, showing that the electric field plays an important role in the crystallization process.

6.3.2 Scanning electron microscopy (SEM)

Several works involving the evaluation of edible films have used SEM when trying to correlate the properties of films with the same morphological structure (Chen et al., 2009; García et al., 2009; Rhim et al., 2006). Also in the present work the surface and cross-section morphology of chitosan films were analyzed using SEM.

The chemical characteristics of chitosan and its cationic character offer good opportunities to take advantage of electronic interactions with numerous compounds during processing and to incorporate specific properties into the material. When their film-forming solutions were submitted to an electric field, chitosan films have shown crystals in their structure, evidencing that there must have been morphological influences from that treatment. SEM analyses indicate that the electrical treatment significantly modified the structure of the films towards a more regular structure, which may presumably be reflected on the changes observed in the surface morphology of the film. The surface of the composite control films (Figure 6.2a) looked more homogeneous than that of the treated films (Figure 6.2c and d). Also, a structure with more regular layers was detected in the case of the film produced with the electrically treated film-forming solutions, as can be observed in the cross-section images of Figure 6.3. Lei et al. (2007) mentioned that the major advantage of ohmic heating when preparing film-forming solutions is that the heat is dispersed uniformly throughout the whole liquid compared to water bath heating, concluding that the film formation rate was higher when ohmic heating was applied.

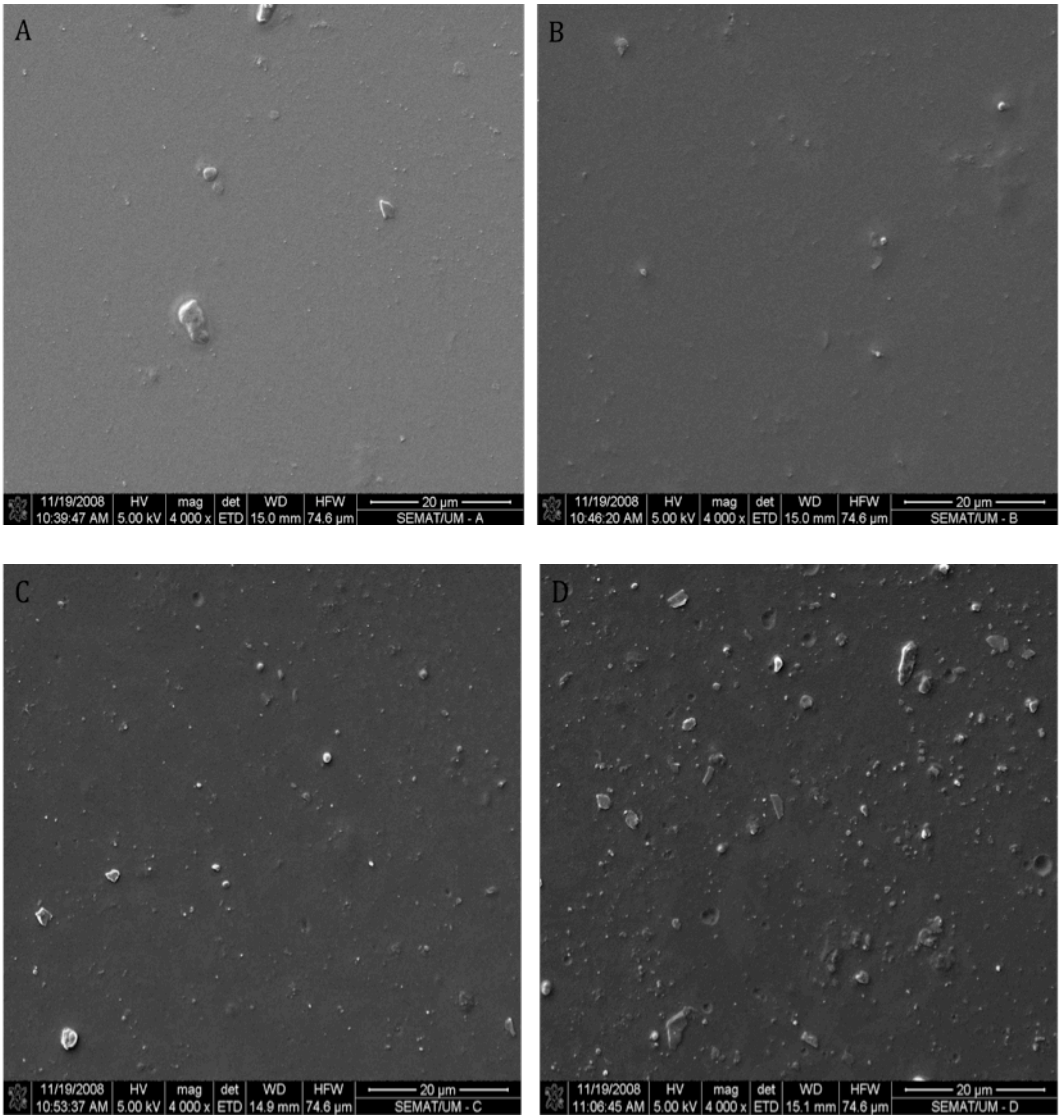
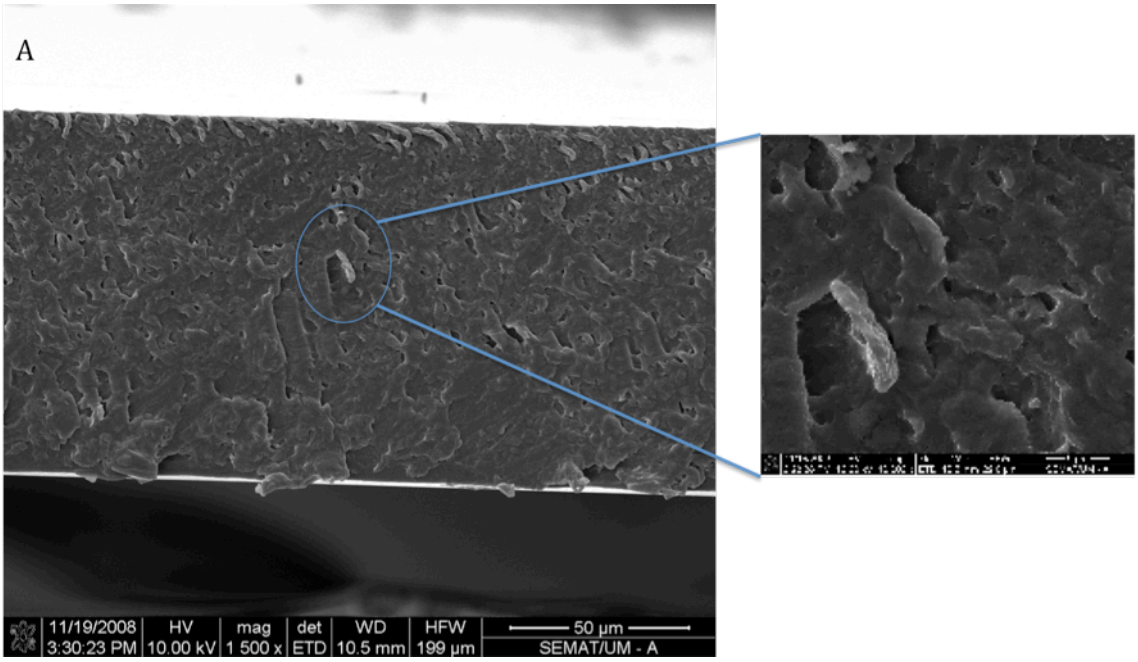


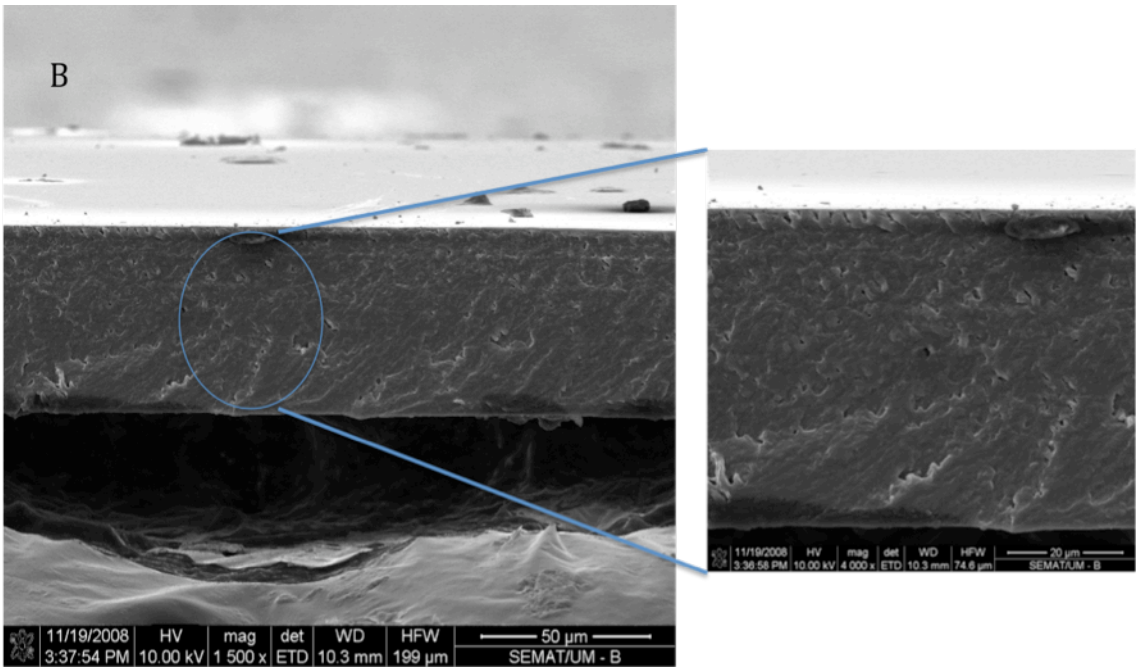
Figure 6.2 - SEM micrographs of chitosan films for (A) control (no heating), (B) conventional heating, (C) electric fields at 100 V cm^{-1}, (D) electric fields at 200 V cm^{-1}.

When studying gas permeation properties of chitosan films Souza et al. (2009) have shown that during the heating process heat was applied uniformly to the whole volume of the film, accelerating the collisions between molecules. This process can provide an improvement in the crystallinity of the chitosan film, thus increasing the material's resistance to gas permeation.

The development of films with a uniform and compact layer can be an important achievement towards the improvement of various film properties, such as their permeability to gases. The search for homogeneous structures thus becomes a target of the research involving edible films.

In this perspective, the application of electric fields may provide an interesting solution for that problem and has gained importance in this area of research. Garcia et al. (2009) showed that the surface morphologies of films were influenced by the preparation method, indicating that the application of an electric field during drying was an influencing factor.





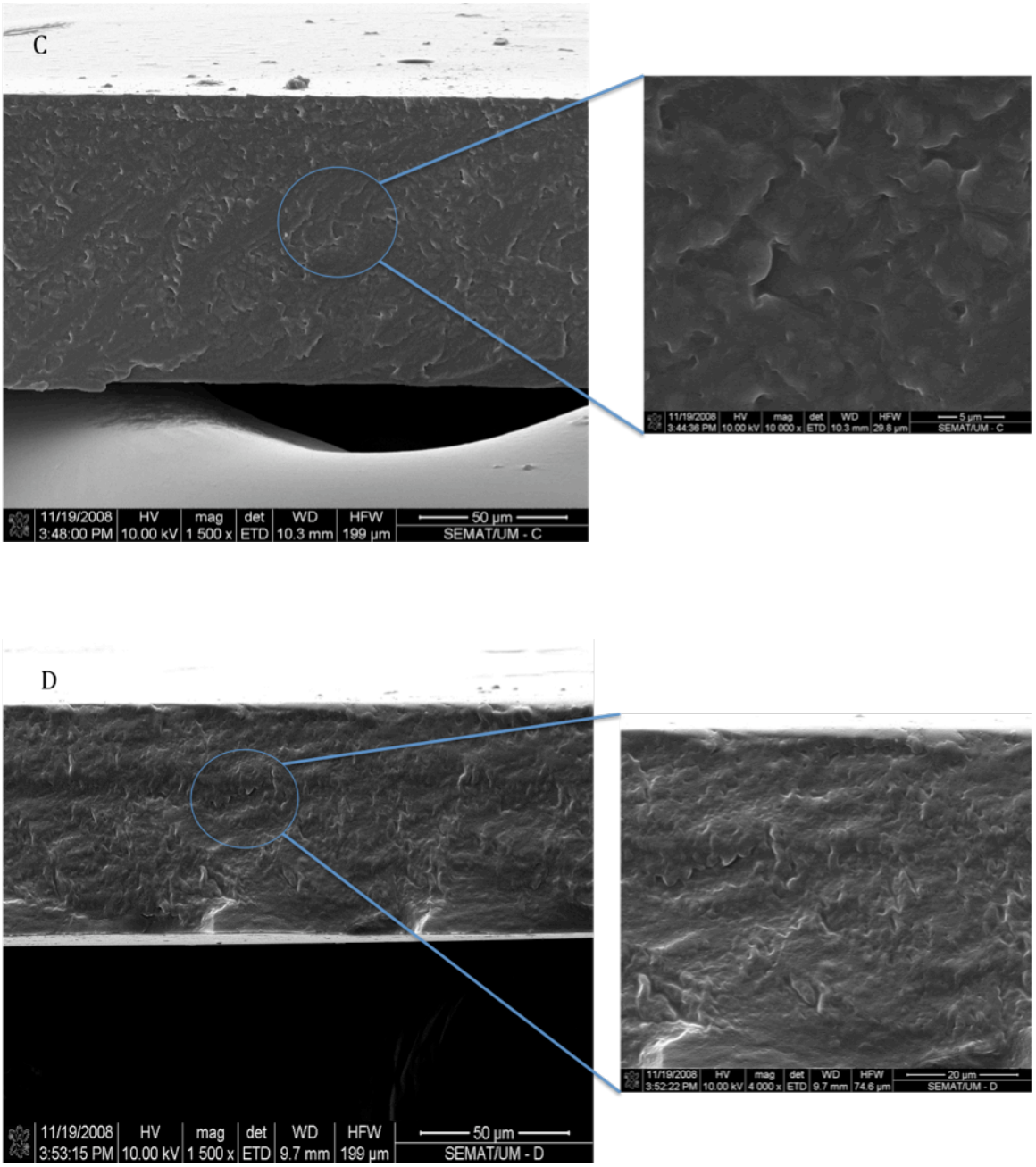


Figure 6.3 - Cross-section of chitosan films for (A) Control (no heating), (B) conventional heating, (C) electric fields at 100 V cm^{-1}, (D) electric fields at 200 V cm^{-1}.

6.3.3 Mechanical properties

Tensile strength *(TS)* and elongation-at-break *(E)* are presented here to characterize the mechanical properties of the chitosan films. *TS* is the maximum tension supported by the film until the moment it collapses.

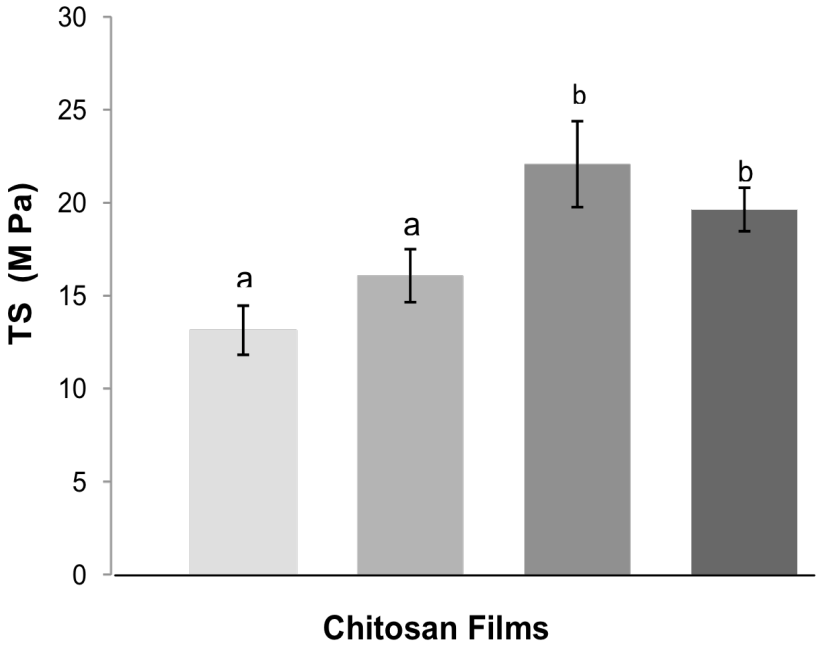
E is a measure of the flexibility of the film and can be considered as a characteristic that defines the ability of the film to deform in place before it collapses. These measurements are important once the mechanical properties of films or coatings depend on the filmogenic nature of the material used, which is directly related to its structural cohesion.

The measurement of the thickness is used to obtain the average thickness of the film, and this value is needed to calculate e.g. the film's mechanical properties. The thickness of films varied between (43 ± 1.37μm and 45 ± 1.03 μm). No significant differences ($P > 0.05$) were detected between control and treated samples and therefore the thickness was presumably not the factor responsible for the differences obtained in the mechanical properties.

The results of the mechanical properties are shown in Figure 6.4. The application of an electric field to chitosan film-forming solutions caused significant differences in *TS* (9 % increase) and *E* (18 % increase).

Several works reported that *TS* of chitosan films increased with increased molecular weight (Park et al., 2002) while other publications refer that the increase of the values of *TS* and *E* for chitosan films is related with the deacetylation degree of the sample (Chen et al., 1994; Ziani et al., 2008). These authors mentioned that those results were due to the higher crystallinity of chitosan films. The polymer chain of chitosan with a higher degree of deacetylation was reported to be more mobile, and this increasing mobility was in turn related with an easier formation of inter-or intra-chain hydrogen bonds. This leads to a higher crystallinity degree that reduces the absorption of water molecules and produces an increase of *TS* (Ziani et al., 2008). In general, the crystallinity of films is related with increased intermolecular forces, thus increasing the rigidity and brittleness of the film (Cervera et al., 2004).

We have observed that when chitosan film-forming solutions were subjected to an electric field, the corresponding films of chitosan showed an increase in the degree of crystallinity of 15.3 % (Table 6.1). These results confirmed the relationship between the application of electric field and the increase of crystallinity. Balau, et al (2004) showed that the electric field plays an important role in the crystallization process, and may also interfere in the water solubility of the films.



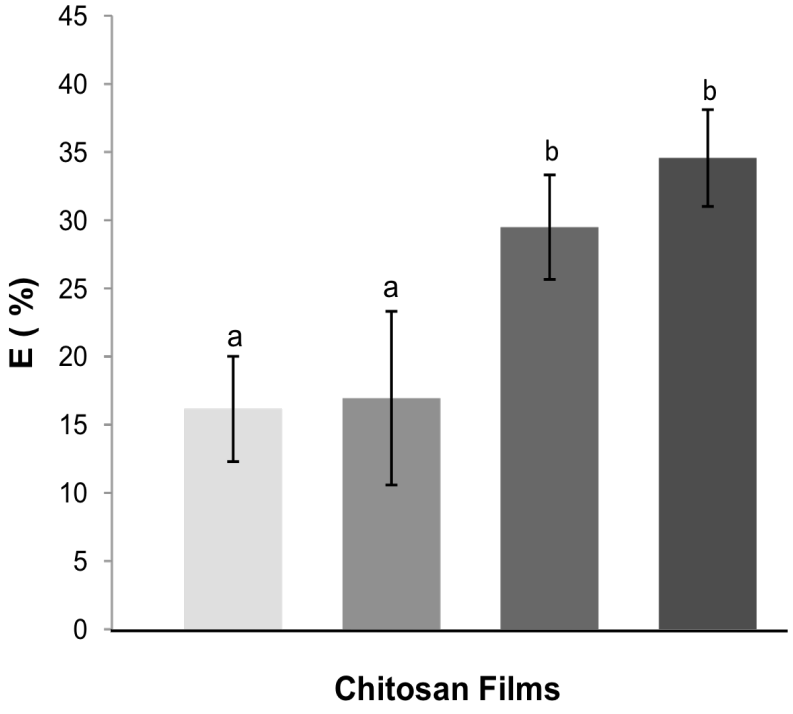


Figure 6.4 - Mechanical properties of chitosan films. (□) Control (no heating), (■) conventional heating, (■) electric fields at 100 V cm^{-1}, (■) electric fields at 200 V cm^{-1}. Different letters in the columns correspond to statistically different samples ($p < 0.05$).

For films in general, mechanical orientation is a common practice to improve gas permeability, optical and mechanical properties; these properties can be modified by controlling chain polymer orientation, resulting on the formation of a more ordered structure, increasing the cristallinity, thus raising the maximum value of *TS* (Kirkwan and Strawbridge, 2003). Garcia, et al (2009), applying the electric field during the drying of films, have shown that electrically treated samples exhibited higher *E* values, indicating that the electrical treatment allowed the alignment of the chains in the field direction, facilitating their stretching and thus increasing their flexibility.

6.3.4 Differential scanning calorimetry and thermal stability

Differential scanning calorimetry (DSC) is a technique which measures the difference between the energy supplied to a sample and the energy supplied to a reference material, when both are subjected to a controlled temperature programming. This is related with the change in enthalpy (ΔH) suffered by the sample. Changes in enthalpy are called first-order transitions (fusion, crystallization, vaporization, adsorption and solidification).

Table 6.1 - Cristallinity, DSC data for the different treatments

Treatments	***Cristallinity index (%)***	***T_m (º)***	***ΔH_m (J/g)***
Control	39.1	122.1±4.50^{a}	21.8±0.46^{a}
Conventional heating	40.4	128.8±1.43^{a}	23.7±0.70^{ab}
100 V·cm^{-1}	52.2	133.1±1.19^{b}	24.8±1.56^{b}
200 V·cm^{-1}	54.4	136.3±2.28^{b}	26.3± 1.85^{b}

*Different letters in the same column correspond to statistically different samples ($p < 0.05$).

In the present work two endothermic peaks were detected for all films. The first endothermic peak that occurred over the temperature range of 78–94 °C was attributed to solvent evaporation (Casariego et al., 2009; Lim and Wan, 1995; Xu et al., 2006), while the peaks in the range of 100–190 °C were attributed to melting transition. The thermal properties of the tested chitosan films are summarized in Table 6.1. The films obtained with electrical field treatment have shown values of the melting temperature T_m and melting enthalpy (ΔH_m) higher than the control. This could be attributed to crystallization of the chitosan films, indicating that the crystal forms and structure of chitosan were changed. Results show that the T_m and ΔH_m increased from 122.1 to 136.3°C and from 21.8 to 26.3 J/g, respectively, with the intensity of the electrical field treatment, suggesting that the degree of crystallinity of chitosan was incresed, in line with the results from the XRD (Table 6.1).

6.4 Conclusions

The application of moderate electric fields to chitosan film-forming solutions had significant effects on the crystallinity index (*CI*), as measured by DSC: those films treated with electrical fields featured higher *CI* values. SEM results also evidenced that the surface of films presented morphological changes, resulting in a more regular structure. Changes were also noticed in terms of *TS* (increase of c.a. 9 %) and *E* (increase of c.a. 18 %).

The development of films with a different structure can be an important achievement towards the improvement of various film properties, such as their permeability to gases. The application of electric fields may provide a novel method for production of films with taylored properties, however further research is needed for a clearer understanding of the importance of these changes on real food systems applications.

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Chapter 7 - Effect of chitosan-based coatings on shelf-life of salmon (*Salmo solar*)

7.1 Introduction

Fresh fish is among the most perishable food products, and the monitoring and control of fish quality is one of the main goals in the fish industry. Postharvest biochemical and microbial changes in fish tissue depend very significantly upon the factors that control the concentration of substrates and metabolites associated with microbial contamination, and the conditions after catching (Duran et al., 2008). Inadequate post-capture handling induces both microbial and endogenous enzyme activities and muscle autolysis leading to protein degradation and loss of functionality. In particular, seafood products contain high levels of polyunsaturated fatty acids that are easily attacked by oxygen-derived free radicals, resulting in lipid peroxidation and meat rancidity (Huang and Weng, 1998). Furthermore, lipid oxidation causes fish rancidity, rendering the product unacceptable for human consumption.

Many indices have been used for the measurement of fish quality during storage. Spectrophotometric detection of the malonaldehyde-thiobarbutiric acid (TBA) complex has been widely used for measuring lipid oxidation in food and biological tissues (Esterbauer and Cheeseman, 1990; Kwon and Veen, 1968; Kwon, 1968). Changes in the microbial population, chemical changes, including Trimethylamine (TMA) content and total volatile base nitrogen (TVB-N) have been proposed as indices of deterioration of fish quality (Anderson, 2008; Kilincceker et al., 2009). Once endogenous enzymes in fish degrade adenine nucleotides during the early stages of the storage period (Boyle et al., 1991), the determination of adenine nucleotides and their degradation products has also been used as a chemical index of fish freshness. In postmortem fish muscle, degradation of adenosine triphosphate (ATP) takes place according to the following sequence: ATP→ADP→AMP Inosine monophosphate (IMP) →Inosine (HxR) →Hypoxanthine (Hx) (Lakshmanam and Gopakumar, 1999). The *K*-value, defined as the ratio of the sum of inosine and hypoxanthine to the sum of ATP and related catabolite compounds, expressed as percentage, is used extensively as a commercial index for estimation of fish freshness (Saito et al., 1959).

The increasing demand for fresh refrigerated seafood with an extended shelf-life has intensified the search for technologies that support fresh fish utilization, being numerous the studies currently focused on using natural ingredients to enhance fish quality and shelf life (abbas et al., 2008). One of the main developments is the utilization of edible coatings for food packaging, designed to replace, at least partially, the use of chemical preservatives.

Several authors have reported the use of chitosan as an antimicrobial and antioxidant agent in muscle foods (Darmadji and Izumimoto, 1994; Gomez-Estaca et al., 2007; Kim and Thomas, 2007).

Moreover, chitosan also has the potential of being applied for food packaging, especially in edible films and coatings (Casariego et al., 2008). These films are excellent oxygen barriers and their mechanical properties are comparable to many medium strength commercial polymer films (Butler et al., 1996; Caner et al., 1998; Park et al., 2002).

The coating process involves wetting of the food product by the coating solution, where a penetration of the solution, followed by adhesion between these two commodities may occur. The wetting stage (spreadability) is very important, because if the suitability of the coating for the object to be coated is ideal, the time interval necessary for such an operation is minimal, or, in others words, spreadability is virtually spontaneous (Casariego et al., 2008).

The objectives of this study are to characterize the surface properties of the food to be coated, the wetting properties of the chitosan coatings and to determine the effects of surfactant concentration and polymer concentration on the wettability of chitosan coatings. This characterization is made in order to evaluate the effect of the application of chitosan based coatings in shelf life extension of sliced salmon fillets stored at 0 °C. This evaluation was performed through the measurement of microbiological and physicochemical properties of the salmon throughout storage

7.2 Materials and methods

7.2.1 Coating materials

The materials used to prepare the edible coating solutions were chitosan (from lobster of the Cuban coasts) obtained in the Pharmaceutical Laboratories Mario Muñoz, Cuba, with a degree of deacetylation of 90 %, Tween 80 (Acros Organics, Belgium) as surfactant, lactic acid 90 % (Merck, Germany) and distilled water.

7.2.2 Preparation of samples

Atlantic salmon (*Salmo solar*) was obtained from a local market in Braga, Portugal. The salmon samples were de-headed, gutted, filleted and cut into slices (200 g average weight slices). Salmon slices were then transported to the laboratory in polystyrene boxes with an appropriate quantity of flaked ice.

7.2.3 Coating solutions

The coating solutions were prepared dissolving the chitosan (1.0, 1.5 or 2.0 % w/v) in a 1 % (v/v) lactic acid solution with agitation using a magnetic stirrer during 2 h at room temperature (20 °C). Tween 80 was added as a surfactant at concentrations between 0.1 % and 0.2 % (w/v) (Casariego et al., 2008).

7.2.4 Critical surface tension

Zisman's method is applicable only for low energy surfaces; therefore it is necessary to determine the surface energy of salmon slices.

For a pure liquid, if polar (γ_L^p) and dispersive (γ_L^d) interactions are known, and if θ is the contact angle between that liquid and a solid, the interaction can be described in terms of the reversible work of adhesion, *Wa*, as: (see equation 4.1 chapter 4)

The contact angle determinations of at least three pure compounds: bromonaphthalene (Merck, Germany), formamide (Merck, Germany) and ultra pure water, on the surface of the salmon (salmon fillet) combined with the values presented below, will allow the calculation of both the independent variable, $\left(\sqrt{\frac{\gamma_L^p}{\gamma_L^d}}\right)$, and the dependent variable, $\left(\frac{1+\cos\theta}{2}\cdot\frac{\gamma_L}{\sqrt{\gamma_L^d}}\right)$, from equation 4.2 (Chapter 4).

The estimation of the critical surface tension (γ_c) was performed by extrapolation from Zisman plots (Zisman, 1964), being the critical surface tension (γ_c) (see equation 4.5 chapter 4).

7.2.5 Wettability

Wettability was evaluated by determining the values of the spreading coefficient (*Ws*) and the works of adhesion (*Wa*) and cohesion (*Wc*). The surface tension of the coating solution was measured by the pendant drop method using Laplace-Young's approximation (Song and Springer, 1996). The contact angle (θ) of a liquid drop on a solid surface is defined by the mechanical equilibrium of the drop under the action of three interfacial tensions: solid-vapor (γ_{SV}), solid-liquid (γ_{SL}), and liquid-vapor (γ_{LV}). The equilibrium spreading coefficient, *Ws*, is defined by Equation 4.6 (chapter 4) and can only be negative or zero (Rulon and Robert, 1993).

Where *Wa* and *Wc* are the works of adhesion and cohesion, defined by Equation 4.7 and Equation 4.8 (chapter 4), respectively.

Contact angle (θ) and liquid-vapor surface tension (γ_{LV}) were measured in a face contact angle meter (OCA 20, Dataphysics, Germany). The samples of the coatings were taken with a 500 mL syringe (Hamilton, Switzerland), with a needle of 0.75 mm of diameter. The contact angle at the fish surfaces was measured by the sessile drop method (Kwok and Neumann, 1999). Measurements were made in less than 30 s. Twenty replicates of contact angle and surface tension measurements were obtained at 21.1 (± 0.4) °C.

7.2.6 Coating treatment and fish samples application

Based on the wettability test the best chitosan coating-forming solution was selected. This selection is made according to the values of (*Ws*) being the better the closer to zero. This coating solution was used to dip salmon fillets and named "treated I". A second batch of the same chitosan solution was submitted to an electric field treatment using field strength of 100 V cm^{-1} "treated II" and also applied on salmon fillets surface through dipping. Coating solutions were previously sterilized under UV light (254 nm) during 2 min prior to being applied to fillet samples. Each fillet sample was dipped into the corresponding treatment solution for 10 s and dried for 1 min on a sterile stainless wire mesh screen. The sample control group was given a similar treatment but without coating application. The fillets salmon (n= 3) for each treatment, were individually packed in plastic and stored at 0 °C for 18 day.
Fish samples were taken from the pack and analyzed microbiologically and physico-chemically at regular intervals (0, 3, 6, 9, 12, 15 and 18 days).

7.2.7 Chemical analyses

7.2.7.1 pH measurement

10 g of each sample (fish muscle) were blended with 100 mL of distilled water in a blender for 30 s and the mixture was filtered through Whatman No. 1 filter paper. The pH of filtrate was measured using a digital pH meter (micropH 2002, Crison, Spain).

7.2.7.2 Total volatile base nitrogen (TVB-N) and Trimethylamine (TMA)

Fish extracts for determination of total volatile base nitrogen (TVB-N) and trimethylamine (TMA) were prepared by homogenizing 100 g of fish sample with 200 ml of 7.5 % (v/v) aqueous trichloroacetic acid (TCA) solution in a laboratory homogenizer for 1 min at high speed. The homogenate was centrifuged at 3000 rpm for 5 min and the supernatant liquid was then filtered through Whatman No. 1 filter paper. TVB-N was measured by steam-distillation of the TCA-fish extract, using the modified method of Malle and Tao (Malle and Poumeyrol, 1989). The same experimental procedure of TVB-N was used for TMA measurement (Malle and Tao, 1987). The only difference was the addition of 20 ml of 35 % (v/v) formaldehyde to the distillation tube to block the primary and secondary amines, whilst leaving only the tertiary amines to react. The amount of TVB-N and TMA were calculated from the volume of sulfuric acid used for titration and the results were expressed in mg nitrogen/100 g of sample.

7.2.7.3 Determination of thiobarbituric acid (TBA) value

The TBA-value (as malonaldehyde) was determined colorimetrically by the method of Porkony and Dieffenbacher as described by Kirk and Sawyer, (1991). A portion (200 mg) of sample was weighed into a 25 ml volumetric flask. An aliquot (1 ml) of 1-butanol was added to dissolve the sample. The mixture was made to volume and mixed. A portion (5 ml) of the mixture was pipetted into a dry stoppered test tube and 5 ml of TBA reagent (prepared by dissolving 200 mg of 2-TBA in 100 ml 1-butanol, filtered and stored at 4 °C for not more than seven days) were added. The test tubes were stoppered, vortexed and placed in a water bath at 95 °C for 120 min and then cooled. Sample absorbance (As) was measured at 530 nm. A solution of pure water (5 mL) + TBA reagent (5 mL) was used as reagent blank (Ab). TBA-value (mg of malonaldehyde/kg of tissue) was obtained by:

$$TBA = \frac{50(As - Ab)}{200} \qquad \text{Eq. 7.1}$$

7.2.7.4 Determination of *K*-value

K-value was determined according to the method of Ryder, (1985). Five grams of skinned fillet obtained from the anterior dorsal regions of the fish were used for the analyses. The fish extract was prepared by homogenizing the sample with 25 ml chilled 0.6 M perchloric acid at 0 °C for 1 min. The homogenate was centrifuged at 6000 rpm for 10 min and the pH of the supernatant was adjusted to pH 6.5–6.8 using a 1 M aqueous potassium hydroxide solution (Fluka-Chemika). The potassium perchlorate that precipitated after standing at 2 °C for 30 min was removed by filtration through Whatman membrane filter No. 1. The filtrate was made up to 20 ml and passed through 0.20 μm Millipore membrane. The samples were stored at -80 °C until further use. 20 μL aliquots of these sample extracts were injected into the HPLC (Jasco, chromatograph 2080-PU intelligent pump (Jasco, Tokyo, Japan) equipped with a Jasco 2070-UV intelligent UV-VIS detector (Jasco, Tokyo, Japan) at 254 nm and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan) with a Nucleosil 120-5 C18 (5 μm particle size, Macherey-Nagel, Düren, Germany) column. The separation of the nucleotide products was achieved using a mobile phase of 0.04 M potassium dihydrogen orthophosphate and 0.06 M dipotassium hydrogen orthophosphate dissolved in 1:1 ratio in MiliQ purified distilled water, at a flow rate of 0.8 $ml.min^{-1}$. The peaks obtained from fish muscle extracts were identified and quantified through standard solution curves. ATP breakdown products comprising ATP, ADP, AMP, IMP, Hx and Ino were measured and K-value was calculated using the equation described by (Saito, 1959):

$$K\% = \frac{(Ino + Hx)}{(ATP + ADP + AMP + IMP + Ino + Hx)} \qquad \text{Eq. 7.2}$$

7.2.8 Microbiological analyses

A sample of 25 g was taken aseptically from each fillet, transferred to a stomacher bag and 225 ml of sterilized peptone water (Becton, Dickinson and Company, France) were added. The mixture was homogenized for 2 min with a Stomacher® 3500 (Seward Medical, UK). Samples (0.1 ml) of serial dilutions of salmon slice homogenates were spread on the surface of the appropriate dry media in Petri dishes for determination of the total aerobic plate count (TPC) on Plate Count Agar (Oxoid, CM325), and incubated at 30 °C for 3 days.

Microbiological data were transformed into logarithms of the number of colony-forming units (CFU/g).

7.2.9 Statistical analyses

All measurements were carried out in triplicate. Statistical analyses were performed using Analysis of Variance (ANOVA) and linear regression analysis. The Tukey test (α = 0.05) was used to determine any significance of differences between specific means (SigmaStat, trial version, 2003, USA).

7.3 Results and discussion

7.3.1 Critical Surface Tension and Surface Tension of sliced salmon fillets

The determination of the surface tension usually involves the measurement of the contact angles that several standard liquids make with that surface. The surface energy of the solid surface is then related to the surface tensions of the liquids and the contact angles. This method involves an estimation of the critical surface tension of the surface of the solids studied, by extrapolation from the Zisman plot.

The determination of the surface tension and of the critical surface tension of the salmon allows the characterization of the surface of its fillets.

Surface tension depends on a number of relatively independent forces, such as dispersion, dipolar, induction, hydrogen-bonding, and metallic interactions (Zisman. 1964). The surface of the salmon displays values of critical surface and surface tension of 30.13 ± 0.12 $mN \cdot m^{-1}$ and 60.64 ± 0.47 $mN \cdot m^{-1}$, respectively. The salmon surface is therefore a low-energy surface (< 100 $mN \cdot m^{-1}$) presenting a low dispersive component (18.18 ± 0.41 $mN \cdot m^{-1}$), which evidences its lesser ability to participate in non-polar interactions. Salmon surface showed a higher polar component, (42.46 ± 0.37 $mN \cdot m^{-1}$) which is usually associated with the high water content found in salmon muscle. A surface with these characteristics interacts with liquid primarily by dispersion forces, influencing the effective spreading of the coating on the salmon surface. The compatibility of the polarity (apolar or polar) of the surface and of the coating may play therefore an important role in the wettability of the surface. Additionally, Mikalsky et al. (1997) showed that, apart from proteins present in meat, other components such as fat carbon hydroxides, play an important role in the adhesion mechanism of meat. The muscle of salmonids is very rich in apolar components (e.g., fat), featuring a significant apolar influence.

7.3.2 Wetability of chitosan solution on salmon sliced fillets

The optimization of the coating solutions composition was based on their ability to spread over a surface and can be made considering three parameters: the spreading coefficient and the adhesion and cohesion coefficients (Casariego et al., 2008). The control of adhesion and cohesion coefficients is very important because if the former promotes the spreading of the liquid, the later promotes its contraction (Ribeiro et al. 2007) and an adequate equilibrium between these two forces is necessary. The wettability was evaluated by determining the values of the spreading coefficient *(Ws)*. Wettability is one of the most important properties when evaluating the capacity of a solution to coat a designated surface. The values of *Ws* (the best values being those closer to zero) on the salmon surface were determined for coating solutions with different concentrations of chitosan and Tween 80.

Results in Table 7.1 show that the spreading coefficient (*Ws*) decreased as chitosan and Tween 80 concentrations increased for the surface studied.

Table 7.1 - Spreading coefficient (*Ws*) obtained for the tested chitosan solutions on salmon fillets.

Solution	Chitosan (w/v)	Tween (w/v)	*Ws*
1	1.0	0.0	- 4.73 ± 0.87^{a}
2	1.0	0.1	- 4.86 ± 0.45^{a}
3	1.0	0.2	- 6.21 ± 0.39^{abc}
4	1.5	0.0	- 5.47 ± 0.45^{a}
5	1.5	0.1	- 8.52 ± 0.58^{be}
6	1.5	0.2	- 9.13 ± 0.52^{ce}
7	2.0	0.0	- 13.17 ± 0.98^{df}
8	2.0	0.1	- 9.75± 0.52^{eg}
9	2.0	0.2	- 12.43 ± 0.87^{fg}

Values reported are the mean ± standard deviations (n =30, 95 % confidence interval, at 20.0 ± 1 °C).
Different letters in the same column indicate a statistically significant difference (Tukey test, p<0.05).

The addition of a surfactant to the coating solution reduces the interfacial tension and improves the adhesion on the surface to be covered (Carneiro-da-Cunha et al., 2009). Choi et al. (2002) reported that the addition of 1 % of Tween 80 to a solution of 1.5 % chitosan improved the compatibility of the chitosan coating solution and apple skin. The improvement of *Ws* with the addition of Tween 80 was also shown by (Ribeiro et al. 2007 and Casariego et al. 2008). Tween 80 acts by reducing the superficial tension of the liquid and by increasing the value of *Ws*. However, in this case, Tween 80 did not influence Ws values due to the fact that the surface of salmon fillets is very rich in water and has therefore more affinity for polar components. Although there were no significant differences ($p>0.05$) between the solutions presenting higher values (solutions 1 to 4); therefore, solution 1 with a spreading coefficient (*Ws*) of -4.73 $mN \cdot m^{-1}$, was chosen to be subsequently analyzed and applied on fish fillets.

7.3.3 Chemical analyses

7.3.3.1 pH measurement

The effect of chitosan coating and storage time on the pH of salmon slices during storage at 0 °C is shown in Figure 7.1. The initial pH of the fish sample was found to be 6.3. The application of both chitosan coating treatments ("treated I" and "treated II") indicated no statistically significant effect ($p > 0.05$) in initial pH values when compared with control salmon sample. After 6 days of storage at 0 °C, the pH value of the control slices (6.58) was significantly ($p < 0.05$) higher than that of samples treated with chitosan coating – treated I (6.47) and electrically treated chitosan coating – treated II (6.46). In general, the increase of pH values may be related to the fast spoilage of the product, with formation of alkaline autolysis compounds (nitrogenous compounds) and production of bacterial metabolites in the muscle during the post-mortem period. Fan et al. (2009) studied the effect of chitosan coatings on quality and shelf life of silver carp during frozen storage and concluded that the lower pH of the coated sample can enhance microbial inhibition and contribute to extend the preservation of fish samples by inhibiting the activity of the endogenous proteases.

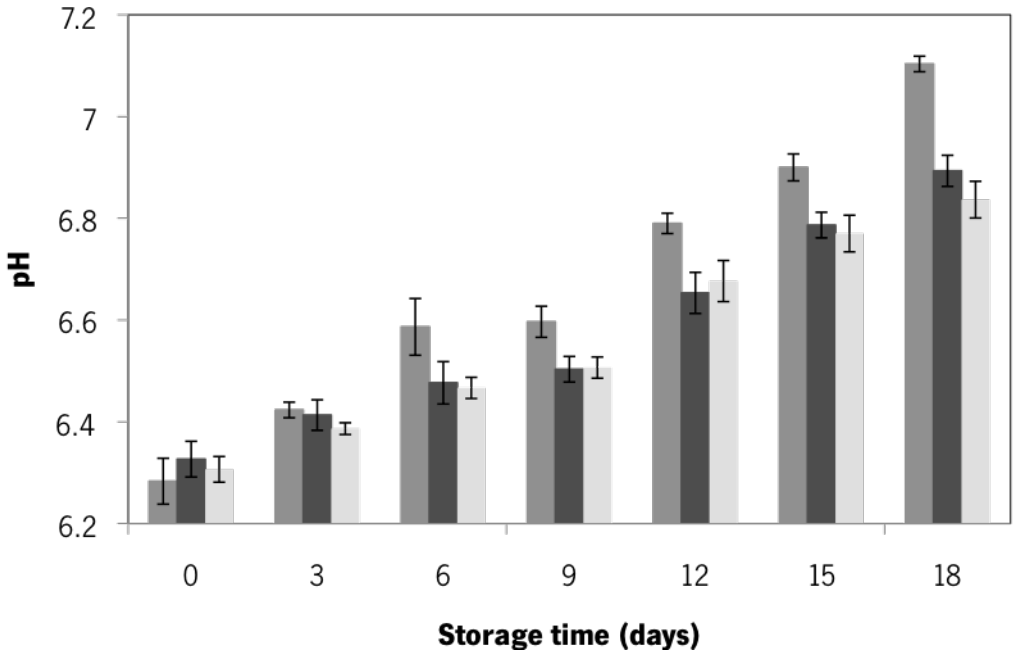

Figure 7.1 - pH values for control (■), treated I (■) and treated II (■) samples of salmon fillet during storage at 0 °C.

7.3.3.2 Total volatile base nitrogen (TVB-N) and Trimethylamine (TMA)

Total volatile basic nitrogen (TVB-N), a parameter that quantifies the compounds composed of ammonia and primary, secondary and tertiary amines, is widely used as an indicator of deterioration of muscle tissues (Fan et al., 2009). The quality and storage life of fish may decrease if they have not been gutted. Fish contains many bacteria in the digestive system and strong digestive enzymes are produced during the feeding periods, which may cause rapid post-mortem autolysis during the later stage of storage. This may give rise to a strong off-flavor, which is often related to the breakdown of protein and the production of nitrogenous volatile materials (Connell, 1990).

Initial TVB-N values (8.05 mgN/100g, 8.00 mgN/100g and 8.06 mgN/100g for control, treated I and treated II, respectively) indicate that the fresh salmon was of good quality, in agreement with the relatively low initial TPC count (3.02 $\log_{10}$ CFU/g). Similar TVB-N values have been found for sliced salmon (Sallam, 2007). TVB-N contents increased gradually, attaining final values of 32.70 mgN/100g, 28.09 mgN/100g and 27.42 mgN/100g for control, treated I and treated II respectively, at the end of storage period (Figure 7.2).

These results may be attributed to the faster reduction of bacterial population or to the decrease of the capacity of bacteria for oxidative deamination (Fan et al., 2009).

Moreover, the differences between control and coated samples can be attributed to the antimicrobial activity of chitosan (Chung et al., 2004).

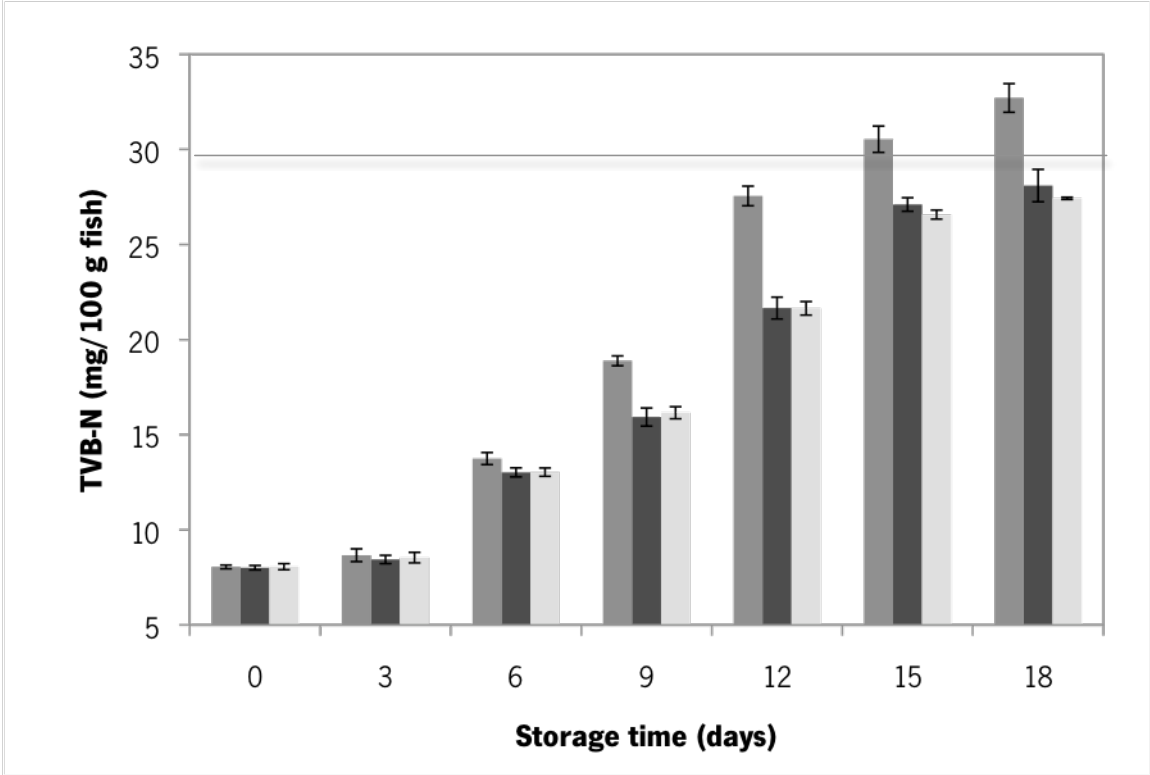

Figure 7.2 - Total volatile base nitrogen (TVB-N) content for control (■), treated I (■) and treated II (■) samples of salmon fillet during storage at 0 °C. The horizontal line represents the rejection limit for fish flesh, which is 30 mg TVB-N/100 g.

TMA and/or nitrogen of trimethylamine (TMA-N) is the most used volatile amine in the fish industry for evaluating freshness and spoilage in marine fish, since it is produced during chilled storage of fish as a result of the bacterial utilization of trimethylamine oxide (TMAO), a naturally occurring osmoregulatory substance found in most marine fish species (Koutsoumanis et al., 1999). Although TMA-N content in muscle is due to bacterial action, its correlation with bacterial growth is not always obtained (Koutsoumanis et al., 1999).

The concentration of TMA-N in numerous fatty fishes never reaches 5 mg TMA-N/100g, a lower value than the rejection limit in fish flesh, which is usually between 5 and 10 mg TMA-N/100g. The concentrations of TMA-N present in the muscle tissue of salmon stored at 0 °C are shown in Figure 7.3. The initial TMA values are 0.87 mg TMA-N/100g muscle, for control sample, 0.91 mg TMA-N/100 g muscle for treated I and 0.95 mg TMA-N/100 g muscle for treated II. A slow increase occurs during the first 3 days of storage, with values of 1.39, 1.33 and 1.38 mg TMA-N/100g being reached.

After 9 days of storage, control samples (without coating) registered higher concentrations of TMA (2.03 mg TMA-N/100g) than treated I (1.80 mg TMA-N/100g) and than treated II 1.70 mg TMA-N/100 g). TMA values of control and both treated samples increased gradually, with values of 6.37±0.14, 5.33±0.29 and 5.12±0.20 mg TMA-N/100 g flesh being obtained for the control and treated samples, respectively, by the end of the storage period (day 18). The observed differences between control and treated samples ($p < 0.05$) may be attributed to fact that chitosan coating inhibits bacterial growth and reduces the formation of TMA, resulting in an extension of shelf-life of sliced salmon. However, samples electrically treated (treated II), indicated no statistically significant effect ($p > 0.05$) when compared with treated I.

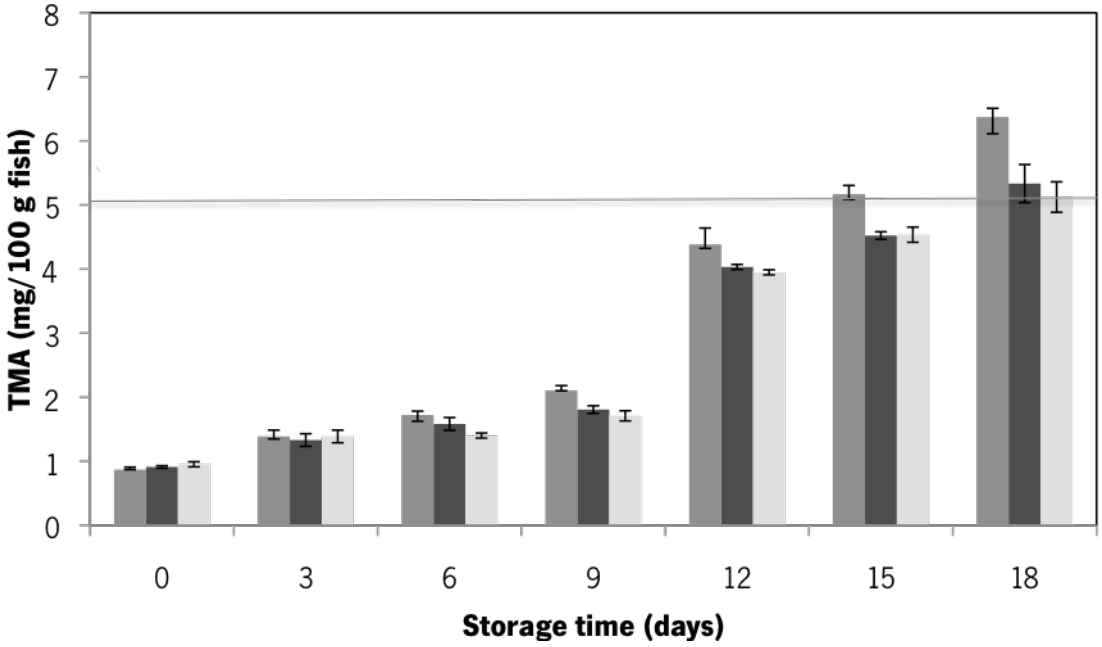

Figure 7.3 - Trimethylamine (TMA) content for control (■), treated I (■) and treated II (■) samples of salmon fillet during storage at 0 °C. The horizontal line represents the lower rejection limit for fish flesh, which is usually between 5 and 10 mg TMA-N/100 g.

7.3.3.3 2-Thiobarbituric acid value (TBA)

2-Thiobarbituric acid (TBA) measures the level of compounds that are responsible for off-flavors/odors and is important during the later stages of lipid oxidation. Lipid oxidation is a second important factor in food deterioration. The TBA value is a widely used index of lipid oxidation, measuring the malonaldehyde (MDA) content (Srikar and Hiremath, 1972). MDA forms from hydroperoxides, which are the initial reaction products of polyunsaturated fatty acids with oxygen (Fernandez et al., 1997).

Changes in TBA value are shown in Figure 7.4. TBA value of the control fish samples was significantly ($p < 0.05$) higher than the corresponding value of the coated samples during storage at 0 °C. However, samples electrically treated (treated II), indicated no statistically significant effect ($p > 0.05$) when compared with treated I.

Fan et al. (Fan et al., 2009) showed that chitosan coating clearly inhibited lipid oxidation in fish flesh during frozen storage. The initial TBA value of the control samples was 0.29 ± 0.02 mg MDA/kg fish, increasing to 1.76±0.04 mg MDA/kg fish after 18 days of storage (Figure 7.4).

The final TBA value of coated samples (treated I) was 1.08±0.04 mg MDA/kg fish and a coated sample electrically treated (treated II) was 1.05±0.05 mg MDA/kg fish after 18 days of storage.

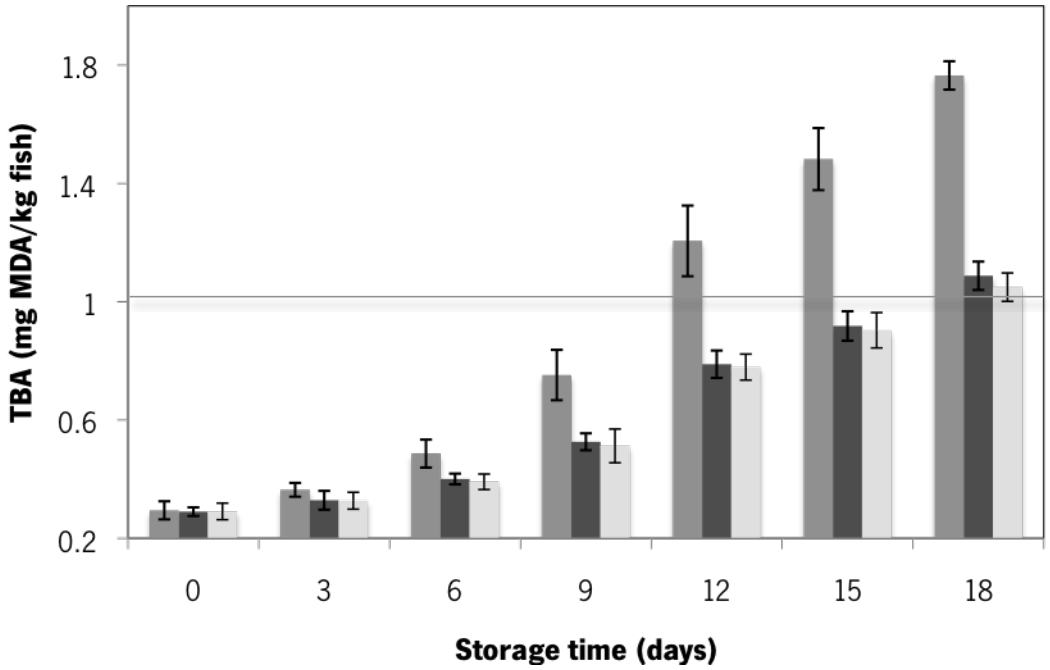

Figure 7.4 - TBA values for control (■), treated I (■) and treated II (■) samples of salmon fillet during storage at 0 °C. The horizontal line represents the lower rejection limit for fish flesh, which is between 1 and 2 mg MDA/kg.

Connel (Connell, 1990) reported that TBA values of 1 to 2 mg MDA/kg of fish flesh are usually regarded as the limit beyond which fish will normally develop an undesirable odor.

The coating process involves wetting of the product to be coated by the coating solution, and possible penetration of the solution into the skin (Hershko et al., 1996), this process being an important factor for the development of an excellent gas barrier. Fan et al. (2009) reported that chitosan coatings reduce the lipid oxidation in fish fillet. Both antioxidant and oxygen barrier properties of chitosan may have contributed to the control of lipid oxidation in salmon fillets. The antioxidation mechanism of chitosan can be explained by the formation of a stable fluorophore resulting from the reaction of primary amino groups of chitosan with volatile aldehydes such as malondialdehyde, which is derived from fats breakdown during oxidation (Weist and Karel, 1992).

In addition, chitosan coatings and films have been reported to be good barriers to oxygen permeation (Casariego et al., 2008). Sathivel et al. (2007) showed that chitosan coating applied on the surface of pink salmon fillets may act as a barrier between the fillet and air surrounding it, thus slowing down the diffusion of oxygen into the fillet.

7.3.3.4 *K*-value

Postmortem degradation of ATP in fish muscle occurs due to endogenous enzymatic activity. This degradation goes through the intermediate products ADP, AMP, IMP, INO and Hx (Church, 1998; Ehira and Uchiyama, 1986; Perez-villarreal and Pozo, 1990). Most of the adenosine nucleotides disappear quickly since they are degraded to IMP within 1-3 days after fish capture, and as the degradation continues, INO and then Hx will be produced (Ehira and Uchiyama, 1986).

K-value, defined as the ratio (x100) of nonphosphorylated ATP-breakdown products by the total ATP-breakdown products, has been used as a freshness measure in fish species (Lin and Morrissey, 1994; Vázquez-Ortiz et al., 1997). Many factors affect the *K*-value of fish, including fish species, type of muscle, stress of fish during capture and storage temperature (Erikson et al., 1997; Guizani et al., 2005).

The *K*-values of salmon were calculated from the concentration of nucleotide over the 18 days of storage (Figure 7.5). Initial *K*-values were 10.6 %, 11.5 % and 11.8 for control and treated samples I and II, respectively. A high *K*-value of 49.7 % at day 18 of storage was obtained for the control sample. Both coated samples (treated I and treated II) presented a significantly (p<0.05) lower *K*–value (46.3 %, 43.6%) when compared with the control at the end of the same period. Therefore, between the samples treated, the treated II (electrically treatment) showed a significantly (p<0.05) lower *K*–value. This observation may be possibly explained by the ability of chitosan to minimize the activity of 5-nucleotidase (Aubourg et al., 2005; Losada et al., 2005). Fan et al. (2009) showed that chitosan was effective in inhibiting the degradation of ATP and extending frozen storage life of fish samples. Freshness indicators related to the breakdown of nucleotides are based on the autolysis of ATP in the muscle.

The rapid rise of *K*-value is entirely due to the sharp decline of IMP in the fish flesh. The loss of IMP through degradation to HxR and Hx would cause loss of fish freshness (Ozogul et al., 2000).

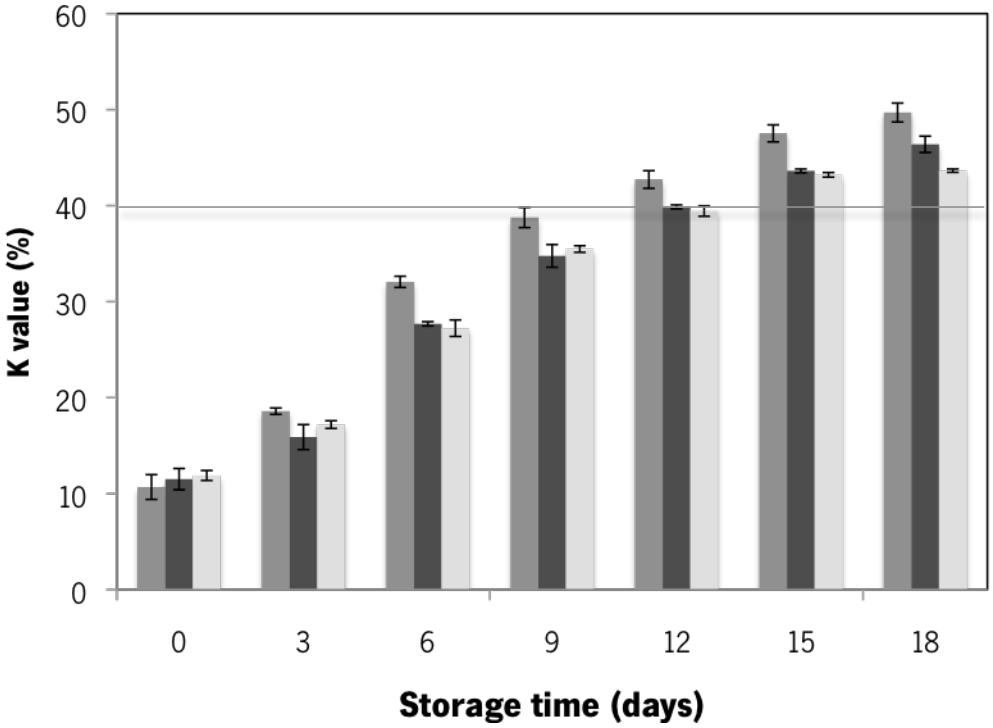

Figure 7.5 - *K*-value for control (■), treated I (■) and treated II (■) samples of salmon fillet during storage at 0 °C. The horizontal line represents the rejection limit for fish flesh, which is 40 %.

7.3.4 Microbiological analyses

The composition of fish flesh "makes" it prone to microbial growth, therefore fish spoiling occurs during storage mainly as a result of microbial activity (Colby et al., 1993). The changes in the microflora of salmon during storage under 0 °C with or without addition of chitosan coating are shown in Figure7.6. A low bacterial load - TPC of 3.02 $\log_{10}$ CFU/g - was obtained at the start of the storage period. Similar low initial TPC values have been reported for fresh fish (Fan et al., 2009).

TPC of chitosan-coated fish samples (treated I and II) were found to be the same as that of the control samples during the first 6 days of storage and the different treatments (treated I and II), indicated no statistically significant effect (p >0.05) simultaneously. However, after day 6, a slower increase in TPC values was observed for coated samples when compared with control, 6.88 $\log_{10}$ CFU/g being obtained for the treated samples (treatment I and II) on the 15th day. This value did not exceed the maximal permissible limit of 7.0 $\log_{10}$ CFU/g, considered as the upper acceptability limit for marine species (ICMSF, 1986), while the TPC of control samples reached 7.05 $\log_{10}$ CFU/g on the 12th day of storage.

These results show that chitosan coating was effective in extending for 3 days the shelf-life of the salmon. These differences may be due to the chitosan antimicrobial activity, which is effectively expressed in aqueous systems (Sudarshan et al., 1992; Wang, 1992). Fan et al. (2009) evaluated the effect of chitosan coating on the quality and shelf life of carp during frozen storage, also demonstrating that chitosan solution coating was effective for extending from 25 to 30 days the storage life of the fish samples at -3 °C.

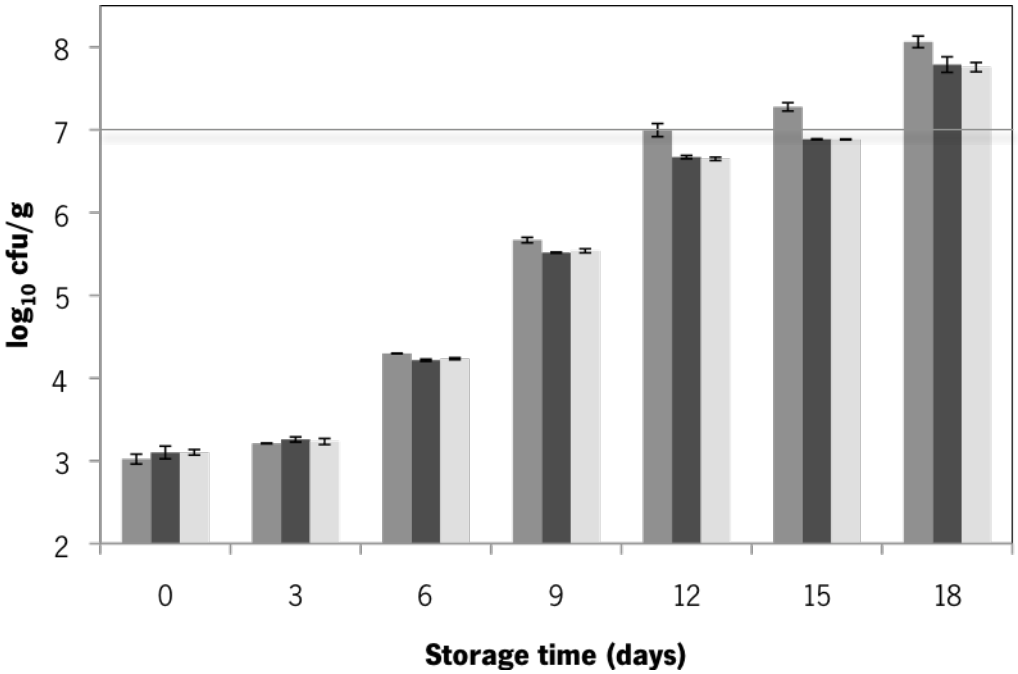

Figure 7.6 - Total aerobic plate count (log CFU/g) for control (■), treated I (■) and treated II (■) samples of salmon fillet during storage at 0 °C. The maximum permissible limit for consumption is 7.0 log10 CFU/g, represented by the horizontal line.

7.4 Conclusions

This work shows how the wettability (*Ws*) can be used as a parameter for coating optimization. The fillet surfaces were found to be of low-energy and therefore Zisman's method was used to determine their wettability. These results show that the application of chitosan coatings on fillet salmon samples stored at 0 °C resulted in a reduction of the microbial count at least after 18 days of storage.

These coatings may act as an additional hurdle to overcome the contamination of salmon, thus improving the microbiological safety of salmon fillets during storage at 0 °C.

Furthermore, edible coatings not only help in retarding the growth of microorganisms but also help in the maintenance of chemical constituents, therefore reducing lipid oxidation. This fact suggests an improved efficiency in lipid oxidation control, thus improving fish quality attributes and extending its shelf life.

This work shows that chitosan coatings can be applied to fresh salmon in order to maintain the chemical quality and extend for three days the shelf-life of the product during storage at 0 °C. Important data have also been generated on the use of edible coatings on fish and, overall, this is relevant information on the use of such materials for application in this type of food.

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Chapter 8 - General conclusions and futures perspectives

The work presented in this thesis is the result of a plan that aimed at studying the chemical characterization of new materials of natural, renewable origins, which can be used as edible coatings for foodstuffs and which can replace with advantages those actually in use. The use of electrical fields treatments on coating-forming solutions was also evaluated, as previous studies pointed at the possibility of changing films' and coatings' properties through the application of an electrical current.
The paragraphs below summarize the main contributions of the present work:

- The sulfated polysaccharide from marine red algae *Gracilaria birdiae* is composed of galactose (65.4 %) and methyl derivatives 6-O-methyl-galactose (9.2 %) and in smaller quantities 3-O- and 4-O-methyl-galactose (0.33 %). This polysaccharide also presents a high content of 3,6-anhydrogalactose (25.6 %) and has a sulfate content of 8.4 %. The sulfated polysaccharide of Gb characterized by FTIR exhibits the characteristic bands of agarocolloids (at 1375 and 770 cm-1). It has also been shown that the *G. birdiae* sulfated polysaccharide is a promising agent to be evaluated for the application in the food industry, and that it presents a significant antioxidant activity.

- The cheese surfaces were found to be of low-energy and therefore Zisman's method was used to determine their wettability. Cheese has the ability to participate in non-polar interactions, as a consequence of the higher values of the dispersive component. The best values in terms of Ws were obtained for cheese with the following formulations, 0.5 % of polysaccharides of *G. birdiae*, 2.0 % of glycerol; 1.5 % of polysaccharides of G. birdiae, 0.5 % of glycerol and 0.5 % of oil; and 1.5 % of polysaccharides of *G. birdiae*, 0.5 % of glycerol/sorbitol and 0.5 % of oil. This procedure is important in order to ensure that the application of the coating solutions on the cheese is made uniformly and easily, in view of future industrial uses. These coatings showed lower values of O_2 permeability, which are important once the oxygen in contact with the cheese contributes to lipid oxidation and to the growth of undesirable microorganisms.
  In terms of *WVP*, lower values of *WVP* were obtained. This characteristic is important in the maintenance of water content, therefore reducing cheese weight loss throughout storage, and suggests an improved efficiency in water loss control, thus improving cheese quality attributes and extending its shelf life. Decreasing the light incidence on the cheese (light promotes fat oxidation) is another important achievement and interesting high values of opacity were

obtained. These findings provided important information on properties of *Gb* agar films in view of their use by the food industry e.g., as coatings and films for the improvement of food storage conditions.

- The results obtained showed that the application of a moderate electric field to the film-forming solutions has statistically significant effects on the film´s physical properties and structure. In general, the most pronounced effect of the field strength was observed for treatments made at 100 $V \cdot cm^{-1}$ or higher. The solubility in water and the water vapour, oxygen and carbon dioxide permeability coefficients showed a positive correlation with the application of an electric field. In practice, the changes in the film properties induced by the application of the electrical field may translate into an improved shelf-life of the products due to reduced water loss (calculated on the basis of the lower *WVP* values achieved) and reduced O_2 and CO_2 exchanges (due to the lower values of O_2P and CO_2P), which will mean a slower metabolism e.g. in fruits and vegetables (Casariego et al., 2008). Future work should be directed towards the confirmation of these effects in real food systems.

- The application of moderate electric fields to chitosan film-forming solutions had significant effects on the crystallinity index (*CI*), as measured by DSC: those films treated with electrical fields featured higher *CI* values. SEM results also evidenced that the surface of films presented morphological changes, resulting in a more regular structure. Changes were also noticed in terms of *TS* (increase of c.a. 9 %) and *E* (increase of c.a. 18 %).

  The development of films with a different structure can be an important achievement towards the improvement of various film properties, such as their permeability to gases. The application of electric fields may provide a novel method for production of films with taylored properties, however further research is needed for a clearer understanding of the importance of these changes on real food systems applications.

- This work shows how the wettability (*Ws*) can be used as a parameter for coating optimization. The salmon fillet surfaces were found to be of low-energy and therefore Zisman's method was

used to determine their wettability. These results show that the application of chitosan coatings on fillet salmon samples stored at 0 °C resulted in a reduction of the microbial count at least during 18 days of storage. These coatings may act as an additional hurdle to overcome the contamination of salmon, thus improving the microbiological safety of salmon fillets during storage at 0 °C. Furthermore, edible coatings not only help in retarding the growth of microorganisms but also help in the maintenance of chemical constituents, therefore reducing lipid oxidation. This fact suggests an improved efficiency in lipid oxidation control, thus improving fish quality attributes and extending its shelf life.

It is also shown that chitosan coatings can be applied to fresh salmon in order to maintain the chemical quality and extend for three days the shelf-life of the product during storage at 0 °C.

Important data have also been generated on the use of edible coatings on fish and, overall, this is relevant information on the use of such materials for application in this type of food.

Some suggestions for future work are presented below:

- Further antioxidant activity analysis of the sulphated polysaccharide from *Gb* must be tested. The sulphated polysaccharide structure from *Gb* could be described through molecular modelling techniques, as a further step to elucidate the structural x activity relationship in this class of antioxidant polysaccharide.

- Evaluate with different temperatures, the use of previously optimized sulphated polysaccharide coatings, to decrease the O_2 consumption and the CO_2 production rates to improve the cheese shelf live.

- A more complete study should be developed to better understand the mechanisms of solutes transfers through polymers from agricultural sources, such as edible films and coatings.

- Study and characterize the molecular structure of polymers when subjected to electric fields through molecular modelling techniques, as further step to explain the molecular changes obtained during process.

- Electric field provides a novel method for production of chitosan films with distinctive properties. However further research with different polymers is needed for a clearer understanding the importance of these technique in different edible coatings. Application in different food systems should also be tested.

- Further studies in the application of sulphated polysaccharide coatings in food such as tropical fruits and minimally processed fruits, etc., could be also studied.

- Study the formulation of edible coatings or films on a commercial scale.